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Editorial

It will come as no great surprise to most doctors who work in hospital that the Editorial Board should have thought it timely to devote a number of the Journal to the problems of hospital infection, stressing the lessons which can be learnt from the emergence of the resistant staphylococci and the coliforms as the main organisms responsible for recent hospital epidemics.

Most of the experts who were invited to participate in producing this number have emphasized that few really new problems have to be faced. The use of antibiotics, sometimes woefully indiscriminately, having led to the emergence of resistant strains of common bacteria, it now behoves us to reassess basic principles of asepsis and antisepsis. The contributors to this number thus scrutinize a wide range of hospital methods which, though greatly improved in recent years, are known to be associated with primitive practices which may result in bacterial contamination. Dirty ward towels, primitive ward sweeping, and inadequate sluices and toilet accommodation are almost bound to cause failures and produce high infection rates. A single failure may result in an individual patient acquiring an added hospital infection but constant disregard of the principles of control of infection may lead to tragedies in hospital communities.

Failures were exposed perhaps more glaringly after the first flush of antibiotic successes. Adventurous surgeons were enabled to undertake the most extensive and courageous surgery, and great advances were made under the umbrella of one or more antibiotics. The spread of the belief in antibiotic safeguards led to a relaxation in techniques in many procedures because the treatment of the subsequent infection was simple. The price is now being paid, and the race between the discovery of new antibiotics and the emergence of other resistant bacterial strains is thrilling but alarming. Even the discovery of the nucleus of penicillin does not give us the same confidence in the future control of bacterial infection as could once have been hoped.

This volume is intended therefore to act as a reminder and an encouragement to those who should be responsible for the bacteriological safety of patients in hospitals. Simple daily procedures, such as floor cleaning, hand washing, ward and theatre routine, and sterilizing methods, are reexamined and recommendations are made on many of these techniques. The reader will no doubt agree after reading the articles on the autoclave, for example, that there is a responsibility on the part of doctors and of managements not only to install machines which work, but to place the responsibility for their continued efficiency on specified and competent shoulders.

The authors have also put forward views on such future prospects as prepackaged sterilized and disposable materials for general ward use, and sterilization of other fabrics and instruments by gamma radiation or by ethylene oxide. Perhaps most of all, however, they all stress the need for strict adherence to fundamental principles of medical and nursing techniques if we are to control and combat sepsis in a hospital community.

Several of the articles overlap and appear repetitive. This is inevitable and excusable as each author was asked to review the same subject of sterilization from his own specialist point of view.

Because of the remarkable pioneering work in this field done by Leonard Colebrook while working at Queen Charlotte's Hospital and at the Birmingham Accident Hospital, the Editorial Board felt that they would like to honour him and his work by dedicating this number of the Journal to him.

The Editorial Board hopes that this comprehensive review will be read by all those individuals and bodies—medical, nursing, and administrative—who have to shoulder the responsibility of preventing hospital infection. For this reason extra copies have been printed and can be obtained from the publishers.

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Hospital infection yesterday and today

MARY BARBER

From the Postgraduate Medical School of London

HISTORICAL BACKGROUND

BEFORE LISTER

Cross infection has been a problem throughout the ages wherever the sick have been housed. It was particularly rife in some of the larger hospitals of Europe in the centuries before Lister, making all but the most imperative operations unthinkable. Paré in the sixteenth century complained that infection at the Hôtel Dieu in Paris was so frightful that no operation could rightly be contemplated in that institution.

The graphic accounts of John Bell at the beginning of the nineteenth century paint a horrifying picture.

> 'There is no hospital,' he wrote (1801), 'however small, airy or well regulated, where this epidemic ulcer is not to be found at times; and then no operation dare be performed! every cure stands still! every wound becomes a sore, and ever sore is apt to run into gangrene: but in great hospitals especially, it prevails at all times and is a real gangrene; it has been named the Hospital Gangrene; and such were its ravages at the Hôtel Dieu of Paris (that great storehouse of corruption and disease) that the surgeons did not dare to call it by its true name.'

John Bell saw no solution to the problem.

> 'What then, is the surgeon to do?' he continues. 'Is he to seek for washes or dressings to cure such a disease as this? Is he to expend butts of wine, contending, as it were, against the elements? No! Let him bear this always in mind, that no dressings ever have been found to stop this ulcer; that no quantities of wine or bark which a man can bear have ever retarded this gangrene; let him bear in mind that this is a hospital disease; that without the circle of the infected walls the men are safe; let him, therefore, hurry them out of this house of death . . . let him lay them in a schoolroom, a church, on a dunghill or in a stable . . . let him carry them anywhere but to their graves.'

FROM ANTISEPTICS TO ANTIBIOTICS

This hopeless attitude towards hospital sepsis persisted for another 50 years, until Lister demonstrated its microbial origin and proved to the world that it was a preventable disease. His antiseptic technique was first applied in 1865. The dramatic effec and the consequent revolution in surgery are wel known.

In the years that followed, asepsis largely superceded antisepsis and enormous improvements i hospital, particularly theatre, hygiene were introduced. By about the 1930s the conquest over hospita infection seemed almost complete and operation which would not have been contemplated in the day of Lister became everyday occurrences. Admittedly 'clean' operations still sometimes became infected but this occurred so much less frequently and with so much less severity than formerly that little attention was paid, and few, if any, surgeons kept a record of the incidence.

In the last 20 years antiseptics of a new type have appeared on the scene, namely antibiotics. Unlike the old-fashioned antiseptics, which are general protoplasmic poisons, antibiotics have a selective toxicity for certain cells and are relatively harmless to others. This important property is, of course, the basis of their therapeutic activity, since they will kill bacteria in doses which do not affect the cells of the human body. But the corollary is that no antibiotic yet discovered attacks all species of pathogenic microbe and among sensitive species individual strains may develop new metabolic pathways which bypass the drug's action. Nevertheless the arrival of penicillin in 1940 seemed at first to be the final answer to hospital cross infection, since it was the death warrant of the two organisms at that time largely responsible, *i.e.*, *Strep. pyogenes* and *Staph. pyogenes*.

TWENTY YEARS AFTER

Unfortunately, early hopes with antibiotics have not been fulfilled. The streptococcus has, indeed, lost much of its menace but not so the more adaptable staphylococcus. So far, as will be discussed below, strains of this organism have become resistant to all the antibiotics in common use and appear to flourish in hospitals as never before. Furthermore, cross infection with Gram-negative bacilli is becoming an increasing problem. There is in fact much evidence to suggest that the situation has changed for the worse.

INCIDENCE, SEVERITY, AND BACTERIOLOGY OF HOSPITAL INFECTION TODAY

The exact position with regard to the incidence of hospital cross infection is difficult to assess, because it is subject to wide fluctuations, not only in different hospitals and wards, but even in a single ward in the absence of any obvious changes in technique. Nevertheless there seems little room for doubt that hospital infection with *Staph. pyogenes* and certain coliform bacilli, particularly *Ps. pyocyanea*, has increased in incidence and severity in the last 15 to 20 years (see Hassall and Rountree, 1959; Finland, Jones, and Barnes, 1959).

There are many reasons why this should be so. To begin with, in the first flush of antibiotic triumph, pathogenic bacteria appeared to be defeated and in many, perhaps most, hospitals aseptic technique was relaxed and isolation wards were put to other purposes. Secondly, patients in hospital today are peculiarly susceptible to infection. They tend to be older and many are kept alive for long periods with conditions, such as leukaemia, which predispose towards infection. Moreover, many of the new life-prolonging procedures, such as major operations, intravenous therapy, the prophylactic administration of antibiotics and treatment with corticosteroids and antimitotic drugs, themselves help to create favourable conditions for bacteria. Thirdly, some of the drug-resistant organisms, which at present flourish in our hospitals, appear to be of enhanced virulence. Thus our hospitals are full of patients who are highly susceptible to infection and of microbes which lose no time in taking advantage of the situation.

A general picture of the incidence of post-operative sepsis in British hospitals is given in a recent survey by the Public Health Laboratory Service (1960). In a total of 21 hospitals the average incidence was 9·7%, and ranged from 4·7 to 21·8% in different hospitals. *Staph. pyogenes* was the commonest aetiological agent; it was isolated from 60% of the septic wounds and was the only pathogen in 45%. Coliform bacilli were isolated together with staphylococci from 13% and alone from 17%.

The severity of hospital infection today is indicated by the high incidence of bacteraemia. The most extensive study is that of Finland *et al.* (1959) from the Boston City Hospital, Massachusetts. They recorded a total increase in the incidence of all types of bacteraemia since 1935 and an increase in mortality since 1947. Analysis of the bacteria responsible showed a remarkable change during the period and the results are summarized below.

BACTERAEMIA DUE TO GRAM-POSITIVE COCCI Like other investigators Finland *et al.* (1959) noted an increase in the incidence of staphylococcal septicaemia. Thus there were 70 cases in 1935, about twice this number in 1941, and over 200 in each of the years 1955 and 1957. The increasing incidence occurred throughout the period, except for the early years of penicillin (1941-47) during which time there was a slight decline.

The number of cases due to haemolytic streptococci was approximately 50 in 1935 but fell sharply between 1935 and 1941 and remained very low until 1957 when there was a slight rise. The pneumococcus was the most frequent aetiological agent in 1935 when it was responsible for about 100 cases. Thereafter the incidence of pneumococcal bacteraemia remained high and although there was a slight fall in 1955 and 1957, in each of these years there were more than 50 cases. Only minor fluctuations were noted in relation to *Strep. viridans*. Finally, enterococci were isolated from no cases in 1935, but from 23 to 36 cases each year after 1947.

BACTERAEMIA DUE TO GRAM-NEGATIVE BACILLI The incidence of bacteraemia due to various Gram-negative bacilli showed even more striking changes. In 1935 the total number of such cases was less than 40, and about three-quarters of them were due to *Bact. coli*. In 1947 the total number was about 180, of which 70 were due to *Bact. coli*, 45 to *Bact. aerogenes* (which had not been isolated from any cases in 1935), and 40 to *Proteus*. In all the subsequent years of study these three species were each responsible for from 45 to 60 cases per year. Bacteraemia due to *Pseudomonas pyocyanea* was rare in 1935, but the number rose steadily, reaching 20 cases in 1957.

DRUG-RESISTANT STAPHYLOCOCCI IN HOSPITAL

EMERGENCE OF RESISTANT STRAINS

PENICILLIN AND TETRACYCLINES The story of the emergence of drug-resistant staphylococci in hospitals over the past 20 years is well known. Phage-typing studies have shown that, whatever the ultimate mode of origin of such strains, selection has been the major factor in their increasing incidence in hospital (see Barber, Hayhoe, and Whitehead, 1949; Rountree and Thomson, 1949). With the widespread use first of penicillin in the 1940s and then of the tetracyclines in the 1950s the hospital staphylococci have in fact been passaged from patient to patient in the presence of these antibiotics one after the other.

Staphylococci do not readily become resistant to either penicillin or the tetracyclines and both cross infection and the widespread use of these drugs have played a part in the emergence of strains of staphylococci resistant to them. Even with both factors operating, penicillin-resistant staphylococci were uncommon until penicillin had been freely available in hospitals for several years. By 1946, however, they were becoming quite frequent (Barber, 1947) and a few years later in hospitals all over the world they outnumbered penicillin-sensitive strains (see Barber and Rozwadowska-Dowzenko, 1948; Rountree and Thomson, 1949; Nichols and Needham, 1949 and many others). Today penicillin-resistant staphylococcal infection is common even in hospital outpatients (MacFarlane, Murrell, Shooter, and Curwen, 1960).

The story was more or less repeated with the tetracyclines except that by the time the latter were introduced, most hospital staphylococci were already resistant to penicillin and it was these penicillin-resistant strains which became resistant to the tetracyclines. Thus by 1952, in many hospitals at least, a few strains of *Staph. pyogenes* resistant to penicillin and the tetracyclines were responsible for most of the staphylococcal infections (Clarke, Dalgleish, and Gillespie, 1952; Rountree and Thomson, 1949; Lowbury, Topley, and Hood, 1952; Kirby and Ahern, 1953).

STREPTOMYCIN The story with streptomycin and chloramphenicol, both of which were introduced into hospitals between penicillin and the tetracyclines, has been rather different, simply because for different reasons the use of both drugs has been limited. In the case of streptomycin, this was because early studies proved that nearly all species of bacteria could become resistant to its action with unparalleled rapidity. It is not surprising, therefore, that in spite of restriction in its use, many hospital staphylococci are resistant to streptomycin as well as to penicillin and the tetracyclines.

CHLORAMPHENICOL has been kept in reserve, because of its potential danger to the bone marrow. Before this danger was appreciated chloramphenicol-resistant staphylococci were emerging in many hospitals, particularly in the U.S.A., but when the use of the antibiotic was no longer widespread the trend was reversed (see Kirby and Ahern, 1953) and until very recently chloramphenicol-resistant staphylococci were uncommon. In the last year or two, however, chloramphenicol has been used more extensively on account of its antistaphylococcal properties, and chloramphenicol-resistant strains are beginning to appear.

ERYTHROMYCIN was introduced into hospitals after we had become aware of the problem of drug resistance and in most places has been kept in reserve. This has had the desired effect and staphylococci resistant to erythromycin are not very common. But since staphylococci develop resistance to erythromycin more readily than to any other antibiotic except streptomycin (Hobson, 1954; Garrod and Waterworth, 1956), erythromycin-resistant strains are by no means unknown, even in hospitals where the drug has been used sparingly. Where it has been widely used the spread of erythromycin-resistant strains in the hospital has been rapid in the extreme (Lepper, Moulton, Dowling, Jackson, and Kofman, 1953).

ERYTHROMYCIN-LIKE ANTIBIOTICS Several antibiotics closely similar to erythromycin are in use, of which the most important are spiramycin and oleandomycin. With all three there is considerable cross-resistance. Between spiramycin and oleandomycin this appears to be complete, but Garrod (1957) has shown that cross-resistance between these two and erythromycin may be dissociated. Thus while strains habituated to erythromycin *in vitro* always show cross-resistance to spiramycin and oleandomycin this is not always the case with erythromycin-resistant strains isolated from patients. With another related drug, E129, resistance is more slowly developed but once again there is some cross-resistance with erythromycin.

NOVOBIOCIN Staphylococci become resistant to novobiocin almost, but not quite, as readily as to erythromycin, and resistant strains have been recorded both *in vitro* (Lin and Coriell, 1956; Garrod and Waterworth, 1956) and *in vivo* (Nichols and Finland, 1956).

MULTIPLE RESISTANCE Erythromycin and novobiocin-resistant staphylococci encountered in hospitals are usually also resistant to penicillin and tetracycline, since the former antibiotics are mainly used in the treatment of infections resistant to the two latter.

CELBENIN (BRL 1241) This antibiotic is a 'penicillin' which is resistant to staphylococcal penicillinase although it stimulates production of the enzyme. Staphylococci resistant to benzyl penicillin (penicillin G) are sensitive to it (Rolinson, Stevens, Batchelor, Wood, and Chain, 1960), and early reports indicate that staphylococci are not readily rendered resistant to it by passage *in vitro* (Rolinson *et al.*, 1960; Knox, 1960). It is moreover argued on theoretical grounds that since the only form of resistance to benzyl

penicillin shown by fully virulent staphylococci is that associated with penicillinase production, celbenin-resistant staphylococci will not become a clinical problem.

Two lines, of objection can be raised to this argument. First staphylococci might produce a new or adapted enzyme which inactivates celbenin. This possibility is supported by the fact that celbenin is not wholly resistant to the penicillinase of *B. cereus*. Secondly, celbenin is not equivalent to penicillinase-resistant benzyl penicillin, since penicillin-sensitive bacteria, including staphylococci, are 20 to 50 times less sensitive to celbenin than to benzyl penicillin. Thus is it possible that the mode of action of celbenin differs in some way from that of benzyl penicillin and that bacteria may develop different pathways of resistance.

Whatever the theoretical arguments, in preliminary experiments I have trained two penicillinase-producing staphylococci to be resistant to from 50 to 100 μg./ml. celbenin; neither strain inactivates the antibiotic, but unlike staphylococci trained *in vitro* to be tolerant to benzyl penicillin they have retained the typical cultural appearances of *Staph. pyogenes* and full coagulase and alpha-toxin activity. Whether celbenin-resistant strains will appear *in vivo* remains to be seen, but it is too early to assume that this cannot happen.

OTHER ANTIBIOTICS Two relatively recent anti-staphylococcal antibiotics, vancomycin and ristocetin, so far have a clean record with regard to resistance. This may be partly due to the fact that since both drugs have to be given intravenously and are toxic to the eighth nerve they have been used sparingly, but, at least with vancomycin, laboratory studies have shown that resistance is not readily achieved (Garrod and Waterworth, 1956). Another new antibiotic, kanamycin, is closely related to streptomycin, and although staphylococci do not develop resistance quite so readily to kanamycin as to streptomycin there is some cross-resistance between the two antibiotics.

MODE OF ORIGIN AND FREQUENCY OF DRUG-RESISTANT STAPHYLOCOCCI

DRUG DESTRUCTION Penicillin-resistant staphylococci isolated from infective processes almost invariably owe their resistance to the production of the enzyme penicillinase which inactivates all the penicillins available for therapy (benzyl penicillin, phenoxymethyl penicillin, broxil) except celbenin. Penicillinase production is a relatively permanent property, but many penicillinase-producing strains tend to yield a proportion of cells which have completely lost the capacity (Barber, 1949; Bondi, Kornblum, and De Saint Phalle, 1953; Fairbrother, Parker, and Eaton, 1954). This loss in enzyme production is sudden and complete and suggests spontaneous mutation. It seems likely, therefore, that the gain in enzyme production also occurs by spontaneous mutation, but since the change has not been inequivocally demonstrated *in vivo* or *in vitro* the mutation must be a rare one.

On the other hand, staphylococcal penicillinase is an adaptive enzyme, in the sense that the enzyme production of penicillinase-producing strains is increased by the presence of the substrate penicillin, although penicillin does not initiate penicillinase production *de novo* by penicillin-sensitive staphylococci. Thus the effect of penicillin on the emergence of penicillinase-producing organisms cannot be entirely overlooked.

DRUG TOLERANCE With all other antibiotics, resistant mutants are capable of growing in the presence of unchanged antibiotic. Such strains almost certainly arise by single or multi-step mutation (see Luria and Delbrück, 1943; Demerec, 1945, 1948; Lederberg and Lederberg, 1952). With streptomycin, gross resistance is the result of a single mutation, and the rate of mutation is very high (of the order of 1 in 10^9 or 10^{10} cell generations). With the other antibiotics in common use against the staphylococcus, gross resistance is the result of multi-step mutation involving a number of genes. With erythromycin and novobiocin the resistant mutants are so frequent that a gross change in sensitivity develops after only a few passages in the antibiotic *in vitro* and is often seen in a strain during the treatment of a single patient. With chloramphenicol and the tetracycline antibiotics the mutation rates are much lower. With vancomycin and ristocetin resistant mutants either do not occur or are very rare.

VIRULENCE OF MULTIPLE RESISTANT STAPHYLOCOCCI

Drug-resistant strains isolated *in vitro* often show an associated reduction in rate of growth and sometimes virulence, so that they may be at a competitive disadvantage with drug-sensitive cultures. But the drug-resistant staphylococci selected in hospitals are those which have survived in the tissues of infected patients and which have been passaged in the hospital community. They are thus likely to be of enhanced virulence and with some strains at least this appears to be the case. Certainly all strains responsible for epidemics in hospitals today are drug-resistant and in the large experience of Williams (1959) usually belong to only about six phage types. Thus all hospitals appear to have become breeding

grounds for a few highly virulent strains of staphylococci, and in devising preventive measures it is important to concentrate on these strains. The recent observation of Moore (1960) that these strains tend to be mercury resistant, if confirmed, offers a quick method for their detection.

STAPHYLOCOCCI IN SURGICAL WARDS

Many studies of post-operative infection over the last five years have shown that the staphylococci responsible for nearly all cases of surgical cross-infection are strains resistant to penicillin and tetracycline and belonging to phage group III or type 80, although such strains are relatively uncommon among staff carriers (Alder, Gillespie, and Thompson, 1955; Barber and Burston, 1955; Shooter, Smith, Griffiths, Brown, Williams, Rippon, and Jevons, 1958; Williams, 1959). Often a few strains appear to be responsible for nearly all the infections in a given hospital. Thus in a study by Shooter and his colleagues (1958) of a surgical ward over a period of eight months 186 different types of *Staph. aureus* were isolated but only 13 caused disease and only three were responsible for sepsis in more than one patient.

In a recent investigation of the surgical wards at Hammersmith Hospital (Barber, *et al.*, 1960) lasting more than two and a half years and involving over 5,000 patients, evidence was accumulated to indicate that some of the multiple resistant strains of *Staph. pyogenes* in the hospital had enhanced virulence, both in their capacity to initiate infection and in the severity of the infection produced. Thus the ratio of infections caused by staphylococci sensitive to all antibiotics or resistant to penicillin only was 1·4 whereas with strains resistant to penicillin and tetracycline it was 2·0 and with strains resistant to penicillin, tetracycline, and erythromycin it was 3·0. Moreover all but five of 49 generalized infections seen and all but one of the 24 fatal cases were due to multiple resistant strains, although such strains were only responsible for 50% of the total number of infections.

STAPHYLOCOCCI IN MATERNITY WARDS

Staphylococcal infection in babies in maternity wards differs from that in surgical wards in that the infecting strains are usually penicillin-resistant staphylococci of phage group I. Such strains also predominate among nurse nasal carriers (Alder *et al.*, 1955; Barber and Burston, 1955). In maternity units they are responsible for infection of the anterior nares and skin of healthy babies as well as for most cases of mild neonatal sepsis (Barber, Wilson, Rippon, and Williams, 1953; Barber and Burston, 1955; Anderson and Williams, 1956).

More severe outbreaks of neonatal infection are, however, sometimes due to multiple resistant staphylococci of phage group III (Beavan and Burry, 1956) or type 80 (Rountree and Freeman, 1955).

SOURCES OF PATHOGENIC STAPHYLOCOCCI

PRIMARY SOURCE The most important source of pathogenic staphylococci in hospitals is staphylococcal sepsis. Staphylococci in open lesions are more readily disseminated than carrier strains, and, as indicated by studies in surgical wards, tend to be of greater virulence. Nevertheless staff carriers represent an important source, particularly in maternity units. In most cases the main site of carriage is the anterior nares, but in some cases *Staph. pyogenes* may be persistently present on skin sites, particularly in the perineal region, in the absence of nasal carriage (Hare and Ridley, 1958).

SECONDARY SOURCES From infected patients and carriers staphylococci are readily spread to the environment. Patients with staphylococcal lesions invariably contaminate their bedding and during bed making the staphylococci are widely disseminated throughout the ward. Equally members of the staff who have lesions or are carriers contaminate their clothing, their handkerchiefs, and their hands. Moreover nurses' and doctors' hands or uniform may become contaminated from contact with an infected patient or his bedding.

From any or all of these sources staphylococci may get into the air and dust of wards and operating theatres. As might be expected staphylococci are far more prevalent in the atmosphere of wards than that of operating theatres and wounds are far more often infected in wards than in the operating theatre. On the other hand the important studies of Gillespie and his colleagues at Bristol have shown that theatre infection when it does occur is liable to be more severe (Gillespie, Alder, Ayliffe, Bradbeer, and Wypkema, 1959).

GRAM-NEGATIVE BACILLI IN HOSPITAL

INCREASING INCIDENCE OF GRAM-NEGATIVE INFECTIONS

Long before the age of antibiotics it was known that wounds in hospital might become contaminated with Gram-negative bacilli. Meleney (1948) in a *Treatise on Surgical Infections* analysed the incidence of various bacteria in clean operation wounds during a period of eight years largely before the introduction of antibiotics. *Staph. aureus* was the commonest in-

vader and was found in 36% of the infected wounds. Gram-negative bacilli were isolated from 10%, and consisted of 'Coli' group 6%, *Proteus* 3%, *Pseudomonas* 1%.

In 1944, Miles drew attention to the possible increase in importance of coliform infections now that efficient weapons (sulphonamides and penicillin) had been found for fighting pyogenic cocci. Three years later Florey, Ross, and Turton (1947) published an important study of the incidence and significance of Gram-negative bacilli in wounds of airborne casualties admitted to the Radcliffe Penicillin Unit. All the patients had received prophylactic injections of penicillin for up to three weeks after wounding, so that pyogenic cocci were relatively uncommon. A total of 1,164 swabs were taken at intervals from 63 deep and 10 superficial wounds. The percentage of the swabs yielding various bacteria was as follows:

	Deep Wounds	*Superficial Wounds*
Bact. coli	35·4	11·5
Proteus	36·1	17·2
Ps. pyocyanea	39·2	17·2
Staph. aureus	14·9	44·8
Strep. haemolyticus	4·3	0·6
Clostridia	11·3	0·0
Indifferent cocci	12·4	21·9
Sterile	11·7	17·8

The presence of coliform organisms appeared to be the result of cross infection in the unit. *Bact. coli* was the earliest invader and was present in 50% of all wounds examined in their first week. *Proteus* appeared most often in the second week and *Ps. pyocyanea* in the third. By the end of six weeks, in spite of a no-touch dressing technique, all but one of the 38 still unhealed wounds had been infected with *Bact. coli* and all but six with *Ps. pyocyanea*.

Of a number of agents used for local treatment streptomycin was the most effective against all three species, although Eusol and Milton were almost as effective as streptomycin against *Ps. pyocyanea*. An interesting observation was the effect of contamination with *Staph. aureus*. In many instances there was a steady disappearance of Gram-negative bacilli after the appearance of staphylococci in the wound. If not removed, the main effects of coliform organisms in the wound were lysis of surrounding clot, persistence of discharge, and consequent delay in bony and soft tissue repair.

CHANGING PATTERN OF GRAM-NEGATIVE INFECTIONS

Since the arrival of the broad-spectrum antibiotics there has been a qualitative change in the type of Gram-negative bacilli responsible for infections. Yow as early as 1952 noted a relative increase in the incidence of *Proteus* and *Pseudomonas* infections. Thus a comparison of the Gram-negative bacilli isolated from specimens received in the bacteriological laboratory of a large general hospital in the U.S.A. in June, 1948, and January, 1951, showed that the proportion of *Bact. coli* fell from 50 to 20%, whereas that of *Proteus* rose from 8 to 32 and *Pseudomonas* from 15 to 30. The same investigation recorded 28 infections, including four bacteraemias due to *Pseudomonas*, and 19, with two bacteraemias, due to *Proteus*, occurring in patients under antibiotic therapy. Finland *et al.* (1959) noted a similar increase in the incidence of *Proteus* and *Ps. pyocyanea* in patients with bacteraemia and meningitis.

INCREASING INCIDENCE OF PS. PYOCYANEA IN HOSPITALS

Among the Gram-negative bacilli, *Ps. pyocyanea* is the most serious menace and is beginning to rival *Staph. pyogenes* as the hospital scourge. Not only is it being found with increasing frequency in all kinds of infections in hospital, but the infections tend to be severe and, since there is no really effective chemotherapy, have a high mortality, reaching almost 100% in cases of established septicaemia.

The frequency with which burns become infected with *Ps. pyocyanea* is well known (Colebrook, Duncan, and Ross, 1948; Jackson, Lowbury, and Topley, 1951; Markley, Gurmendi, and Chavez, 1957). Apart from the resultant delay in healing and the failure of skin grafts, in extensive burns, *Ps. pyocyanea* is liable to invade the blood stream and is a common cause of death (Liedberg, Reiss, and Artz, 1954; Markley *et al.*, 1957).

The incidence of *Ps. pyocyanea* in operation wounds has not often been recorded, but many bacteriologists have the impression that it is on the increase. Figure 1 shows the number of patients in a large London hospital from whom *Ps. pyocyanea* was isolated from specimens of sputum or pus (mainly infected wounds) between 1958 and 1960. The analysis was undertaken because a very large increase in the number of specimens yielding *Ps. pyocyanea* was noted in the first quarter of 1958. The biggest change was the number of patients in the medical wards with *Ps. pyocyanea* in the sputum. Thus in the first quarter of 1958 there were two such patients, whereas in the first quarter of 1959 there were 39. During the same period the incidence of wounds infected with *Ps. pyocyanea* in the surgical wards rose from two to 21. In the summer, as might be expected, the number of patients with *Ps. pyocyanea* in the sputum fell; in the following winter the number again increased, but probably because

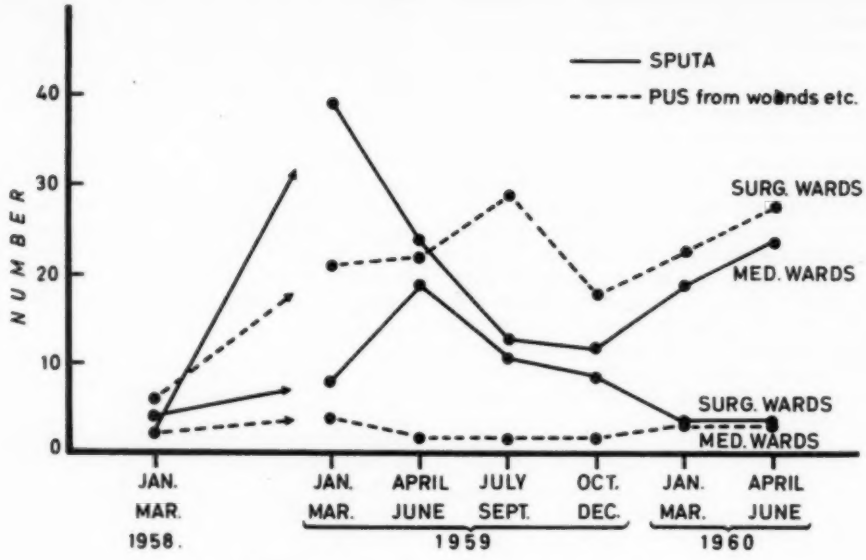

FIG. 1. *Infections due to* Ps. pyocyanea *in a large general hospital.*

the weather was much less severe, only to half the number of the previous winter. Wound infections in the surgical wards have remained between 20 and 30 since the sharp rise in the first quarter of 1959.

Many of the medical patients with pseudomonas chest infections were patients with chronic bronchitis, who had had various forms of chemotherapy and were admitted to the wards already infected with *Ps. pyocyanea*. Since isolation facilities were not available they were admitted to open wards and it seems possible that they acted as a reservoir of infection in the hospital.

PSEUDOMONAS SEPTICAEMIA IN MEDICAL WARDS

In recent years pseudomonas septicaemia has been becoming an increasing problem in medical wards, particularly in units devoted to patients under modern forms of treatment for leukaemia. Forkner, Frei, Edgcomb, and Utz (1958) recorded 23 patients with pseudomonas septicaemia between 1954 and 1957, of whom 22 died. Sixteen of the patients had leukaemia, and of 13 who were studied more carefully, 10 had had previous treatment with antibiotics, eight with steroids, and nine with antimetabolite drugs. The same authors analysed the bacteria responsible for 63 septicaemias in the leukaemia service and found that *Ps. pyocyanea* was second only to the staphylococcus as the aetiological agent.

Williams, Williams, and Hyams (1960) recorded three septicaemias, one chest infection, and one intestinal infection due to *Ps. pyocyanea*, all occurring in a single general medical ward between May and September, 1958. All five patients died and infection with *Ps. pyocyanea* in all cases materially contributed to their death. Of the three patients with septicaemia, two had leukaemia and the third marrow aplasia. All five patients had previously been treated with broad-spectrum antibiotics and all had received corticosteroids.

PRIMARY AND SECONDARY SOURCES OF PS. PYOCYANEA IN HOSPITAL

PRIMARY SOURCE The most important primary source of *Ps. pyocyanea*, as of *Staph. pyogenes*, is infected patients. Unlike the staphylococcus, *Ps. pyocyanea* is rarely carried in the nose or on the skin of healthy individuals, but Lowbury and Fox (1954) isolated *Ps. pyocyanea* from the stools of two out of 64 medical students.

SECONDARY SOURCES Lowbury and Fox (1954) made an extensive study of the distribution of *Ps. pyocyanea* in the environment of a burns unit. They

isolated the organism from the nose and skin of a small proportion of the staff and patients of burns wards, but not from the staff of other wards.

Of the inanimate sources, dust seems to be the most important; 94% of samples from the burns wards and 16% from other surgical wards contained *Ps. pyocyanea*. The percentage of air samples yielding the organism was 86% during the dressing of an infected wound in the burns dressing station, 27% in the burns ward during bed making, and 25% when the ward was at rest. *Ps. pyocyanea* was also frequently isolated from the outside of bandages and the bedclothes of infected patients, and less frequently from objects brought to the patients, such as bedpans, wash basins, food trays, and toys.

In a recently described outbreak, involving eight patients in an orthopaedic ward, infection was traced to contaminated cellulose wadding used for padding plaster casts applied to fractures (Sussman and Stevens, 1960).

PREVENTION

As pointed out by Lister, hospital infection is a preventable disease. That it has not yet been prevented is largely a reflection on the fact that in the past we have underestimated the enemy. If we are to succeed we must attack the problem on many fronts. Different modes of attack and their effects are described in detail in the subsequent articles of this series. Here it is only possible to outline the strategy.

The first line of attack must be on the primary human reservoirs of infection. Infected patients must be excluded from open general wards and the infection controlled, where possible, by means of intelligent chemotherapy. With staphylococci, nasal and skin carriers have also got to be considered. To what extent this would remain necessary once the cycle of cross infection had been broken by the strict isolation of infected patients remains to be determined. But at present there is no doubt that carriers, at any rate of multiple resistant staphylococci, can be a source of danger.

Staph. pyogenes, *Ps. pyocyanea*, and other drug-resistant coliform bacilli in the hospital environment must also be dealt with. This entails rigorous aseptic technique in wards as well as in theatres, clean air, clean bedding, and hygienic methods of dust removal.

Last, but not least, some control of the use of antibiotics in hospitals is essential both to prevent the emergence of drug-resistant bacteria and also to avoid rendering patients more susceptible to infection by such bacteria, through elimination of the normal flora.

Money will have to be spent, but let us bear in mind that at present it is being wasted. In a recent survey by the Public Health Laboratory Service (1960) it was estimated that the cost of post-operative sepsis to the National Health Service is some £5 million per annum, quite apart from the value of any lives lost. To return to the words of John Bell (1801):

> 'No expense should be spared . . . you would willingly expend your own future in such a cause—then do not grudge to employ the revenue of the state for it is employing it and not abusing it! . . . if in the course of a few weeks sixty men die of the disease in your hospital, government has lost a sum which would trebly buy your hospital itself.'

Cross-infection in hospital has remained a problem yesterday and today. If enough people take it seriously there is no reason why it should continue tomorrow.

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A survey of some problems in sterilization

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The methods of sterilization in use in many hospitals in Britain at the present time are inefficient and often unsafe and many so-called sterilizers do not sterilize. In recent years there has been increasing awareness of this unsatisfactory state of affairs. At first a few individuals condemned the situation and showed some glaring examples of breakdown in sterilization (Savage, 1944; Walter, 1948; Howie and Timbury, 1956; Bowie 1955, 1957). More recently, stimulated by the original observations, administrators and hospital authorities are becoming increasingly conscious that the whole machinery of sterilization in hospitals needs a drastic overhaul (Nuffield Provincial Hospitals Trust, 1958; Medical Research Council Working Party's Report, 1959). At the same time many new techniques and methods are becoming available. Rival claims are made for different methods and it is important to keep some sense of perspective and to decide for what purposes different methods of sterilization are most suitable. The aim of this article is to assess the present situation, to outline some of the main problems, and to suggest some of the lines along which development is likely to proceed in the immediate and in the more remote future.

AVAILABLE METHODS OF STERILIZATION

For some articles there are many methods of sterilization available but others can only be sterilized by perhaps one or at the most two different methods. The main methods are:

1 *a*, Dry heat: the conventional hot-air oven, conveyor belt methods, the conducting aluminium block, vacuum ovens; *b*, Moist heat: steam pressure sterilizers, downward-displacement type and high-vacuum type.

2 Chemical methods: liquid disinfectants, gaseous disinfectants, especially ethylene oxide.

3 Irradiation by cobalt 60 or linear accelerator

There are, of course, many special methods, *e.g.*, the use of ultraviolet light for sterilizing surfaces and for attempted sterilization of air, and the use of filtration for sterilizing fluids which cannot be subjected to other more drastic treatments. Special methods of filtration can also be used for the decontamination or attempted sterilization of air. For surface disinfection formaldehyde may be satisfactory, provided it is used under proper conditions of humidity and temperature (Public Health Laboratory Service, 1958). With some types of equipment, *e.g.*, bedpans, adequate disinfection may be achieved by exposure to steam at atmospheric pressure. Finally, for many purposes boiling is a perfectly satisfactory method, but it cannot be too strongly emphasized that many of these methods are only suitable in exceptional circumstances and none can be universally recommended as guaranteeing a sterile product.

GEOGRAPHICAL SCOPE AND LIMITATIONS OF DIFFERENT METHODS

One of the major problems to decide at the present time is to what extent sterilization should continue to be done on a purely local basis and to what extent it should be organized on a larger scale. Until very recently, sterilization has been a very local affair and no large British hospital so far as is known has had a complete central sterile service. Many new hospitals, however, have already constructed or are planning central sterile supply departments. A valuable account of the department already functioning at Musgrave Park, Northern Ireland, has been given by Allison (1960) who also discussed the development and planning of such a service. Surgeons and others in the past have been slow to accept the idea of central sterile supply. They have preferred to have their own instruments sterilized in the immediate neighbourhood of their own theatre. Syringes have been boiled up ready for use in the wards and so on. However, it is now generally agreed that central sterile supply has many advantages over purely local sterilization units scattered throughout a hospital. With the increasing complexity of modern sterilizing plant, it becomes more and more important to get the maximum use out of each unit. This alone is a powerful argument in favour of centralizing sterilization arrangements for any large hospital.

On the other hand there is an increasing feeling that many types of sterilization should not be carried

out on a hospital basis at all but that commercial production and distribution would give more efficient and in the long run more economical results. This new approach is of course closely linked with the increasing production of disposable items of equipment, a trend to some extent forced upon us by the increasing cost of labour and the difficulty of getting efficient washing and sterilization equipment inside the hospital area. With certain types of equipment it looks as though the day of the large central sterile supply department in a hospital is already over before it has begun, and many hospital authorities must be wondering whether it is wise to sink a lot of money in the building of what in a few years' time may be a white elephant. The answer to many of these problems cannot be given at the present time and will come only as the result of experience. The best that can be done is to hope that experience will not be bought at too great a price. For example, it seems fair to say that the supply of disposable equipment sterilized by steam in commercial laboratories and distributed over a wide area is not likely to be an economic proposition. One reason is that the installation of large numbers of autoclaves with the amount of steam required to operate them efficiently is very expensive and the output is rather limited, whereas with other methods of sterilization, such as irradiation, although the capital cost is high, there is every prospect of being able to sterilize large quantities of material economically, for example, rubber gloves and syringes or needles. Of course it is unwise to generalize too much. The relative efficiency and cost of different methods must vary enormously with the nature of the material to be sterilized and with the purpose for which it is required.

METHODS OF STERILIZATION

STERILIZATION BY STEAM UNDER PRESSURE This is a field in which considerable advances have been made in the last few years, not so much because of the discovery of any new principles but because of an increasing realization that principles already well known should be efficiently applied if sterilization by steam is to yield the best possible results. It has been recognized for many years that rapid penetration of steam into the interior of dressings is essential if reliable sterilization is to be achieved. One of the traditional methods of removing air from steam sterilizers has been to make use of a steam ejector to draw out part of the air contained in the cylinder of the sterilizer before steam was admitted, and no sterilizer was regarded as efficient unless it had a 'vacuum' apparatus to remove air. It was, however, pointed out many years ago by Underwood (1934), Savage (1937), and by others that this method even at its best gave only a poor vacuum and could not be expected to do more than remove two-thirds of the air originally present in the sterilizer. Often it was found that it was removing only about one-third of the air. This meant that a great deal of air was still left in the sterilizer. This interfered considerably with the penetration of steam and with the attainment of the required sterilizing temperature and was responsible for many sterilization failures. American opinion then began to stress the desirability of removing air not by this inefficient vacuum system but by an efficient system of downward displacement, and a great deal of American design and practice in hospitals was based on the assumption that the best way of removing air was by downward displacement (Perkins, 1956).

More recently attention has again been drawn to the need for rapid and efficient removal of air by means of the attainment of a really high vacuum, and, especially on the Continent, much progress has been made (Bowie, 1957).

A fresh investigation was made by Knox and Penikett (1958) of the degree of vacuum required in order that steam might penetrate efficiently into a standard drum situated in a particular position in an autoclave. The arbitrary figure of 20 mm. or thereabouts was set as the order of vacuum which must be attained before admitting steam so as to ensure its rapid and reliable penetration. Further experimental data and a full account of this subject were given by Penikett (1960).

Knox and Penikett showed that in the conditions they used it was essential to reach this order of vacuum and emphasized that manufacturers must not be content with any machine which was not capable of rapidly producing it, and subsequent work has shown that with considerable variations in load and packing and with different sizes of sterilizers, this is the kind of vacuum which must be obtained in order to ensure uniformly reliable penetration of steam. The problem which then arose was how best this kind of vacuum could be obtained. Vacua of this order can be obtained by a number of different types of pump, for example, rotary oil pumps, water-ring pumps, and steam-jacketed pumps. Each of these has disadvantages and advantages but the problem has now become one of maintenance and engineering practice, and it is a question of finding out which method is going to give the best results over the longest period of time with the least maintenance trouble and the least cost to hospital authorities.

One point that is still unsettled is the exact time and temperature which should be used for sterilizing different types of equipment. There has been

an unfortunate tendency to link together 'high-vacuum' sterilizers with high-temperature, short-time exposure to steam. It often seems to be taken for granted that the high-vacuum sterilizer should always be operated at a temperature of 134°C. and at about 30 lb. steam pressure. This association is quite fallacious. There is no reason why conventional sterilizing times such as 121°C. at 15 to 20 min. should not be used with the high-vacuum sterilizer just as with the downward-displacement sterilizer. There may be certain advantages in higher temperatures and shorter times. But slight errors in timing become very important if only very short times are used for sterilization and it would seem that a greater margin of safety is present if periods of 15 to 20 min. are used at temperatures of the order of 121 to 125°C. rather than periods of only a minute or two at temperatures as high as 134°C. This again is a question which can be answered only by future experience. At present opinion is divided. For example, we still have not enough information as to the correct time and temperature conditions for the efficient sterilization of rubber gloves by steam under pressure. Until very recently it was accepted that rubber gloves would not stand steam sterilization. Either the gloves were exposed to temperatures which could not possibly be expected to render them truly sterile or if they were exposed to sterilizing temperatures they were damaged beyond possibility of further use. It is now realized that a great deal of the damage to rubber gloves under these conditions was due to the long period of drying which was essential when the downward-displacement method was being used and that the damage was due to oxidation. If high-vacuum methods can be used and if drying conditions can rapidly be established by drawing a vacuum after the sterilization process is over, there seems to be no reason why rubber gloves should not be adequately sterilized either at high temperatures, such as 134°C. for a very short period of time, say three to five minutes, or at temperatures of 121° for 15 to 20 min. Which of these is the better is still uncertain.

STERILIZATION BY DRY HEAT This subject is covered in another article and will not be referred to more than briefly here. Dry heat is used mainly for sterilizing syringes and certain instruments, especially those of which the cutting edge may be damaged by other methods. For syringes, one great advantage of dry heat is that they can be sterilized already assembled and can therefore be relied upon to be sterile inside the container in which they have been assembled so that the risk of contamination before an injection is given is reduced to the lowest possible level. In the conventional hot-air oven sterilization is carried out at a temperature of 160°C. for one hour. Darmady and Brock (1954) have shown that conventional hot-air ovens may vary enormously in their performances and that there is considerable risk of either underheating or overheating, both of which are undesirable. In ovens fitted with efficient fans the temperature is much more uniformly regulated and for many purposes they are entirely satisfactory. Two recent developments are under trial at the present time in various forms of conveyor belt sterilizers and the solid aluminium block which heats the syringes placed in recesses inside the block by direct conduction. The conveyor belt seems to be the most useful apparatus for the large syringe service. The machine can be fed continuously with supplies of syringes, all of which can be guaranteed, provided that the temperature control is satisfactorily adjusted, to receive the same heat treatment as they are carried past the heating units. The aluminium block may perhaps have a considerable future for the small user. It is, of course, not so quick as a small autoclave which can indeed be used for sterilizing syringes but the autoclave has the strong disadvantage that if it is to be reliable then syringes must not be assembled, whereas the aluminium block has the great advantage of sterilizing assembled syringes which need no further handling until the injection is actually given. A syringe service using heat as the means of sterilization has to face competition from at least two other agents, namely, ethylene oxide and irradiation. More will be said about these in later sections. Here, it may be said that syringes sterilized by heat under proper conditions represent probably the most absolute standard of sterility which can be achieved. At the present time the same cannot be said of ethylene oxide except under very special conditions and even irradiation is not an infallible means of producing sterility. At the present time at least one commercial firm is running an efficient syringe service based on sterilization by heat from a series of gas ovens and making use of the conveyor belt principle.

IRRADIATION In recent years increasing attention has been paid to irradiation as a means of sterilizing articles of various kinds. This is being dealt with more fully in another article in this series, but here it may be useful to emphasize some main advantages of the method.

In Britain the method for which most information is available is the use of cobalt 60 as a source. Linear accelerators are also being developed for this purpose. The use of cobalt 60 is only suitable where the process can be carried out on a large enough scale to justify the capital cost and outlay involved.

It must therefore be essentially an industrial process. If, however, this initial cost is accepted, the process has many advantages which would seem to make it almost unique. Articles of very diverse size, shape, and of different materials can be sterilized with complete reliability and very large quantities of material can be handled, giving probably a much greater capacity than that obtainable by any other method with comparable running costs. Materials such as syringes or rubber gloves can be assembled completely packaged and sterilized ready for use. With properly designed plant and flow of materials it can be guaranteed that all parts of the load and all items in it receive a sterilizing dose, and those problems of penetration and uniformity of treatment, which are so important in sterilization by steam and ethylene oxide, do not arise.

GENERAL DISCUSSION

Some of the outstanding problems in sterilization may perhaps best be discussed by posing a series of questions.

1 WHEN IS STERILIZATION NECESSARY? It is necessary here to steer a middle course between two extreme views. The first is that much of the recent interest in sterilization is misplaced and that simple measures such as boiling or the use of chemical disinfectants are perfectly satisfactory for most purposes. Those who hold this view believe that modern developments in sterilization are unnecessarily expensive and do not give an adequate return for the tremendous capital outlay involved. They are supported also by those who believe that central sterilizing services are bad for the morale of nurses and doctors and that it is better that they should be taught how not to sterilize properly rather than to rely on properly sterilized equipment. At the other extreme are those who demand that almost every article of equipment used in hospitals should be sterile. This certainly goes too far in the opposite direction and there is no doubt that there are many articles for which all that is needed is a reasonable standard of hygiene or cleanliness and not sterilization at all. Bedpans, Ryle's tubes, and even much of the water which is used in certain types of operative work might come under this heading. It might in fact be far safer to use good tap water for many of these purposes rather than so-called sterile water from an inefficient tank sterilizer which may be heavily contaminated with *Proteus* or *Pseudomonas*.

It would certainly make the task of hospital administrators a good deal easier if a decision could be reached as to what articles it is really necessary to sterilize. Once this decision is made then it seems clear that the best possible methods must be used for ensuring that they are properly sterilized and maintained in a sterile state. It is quite true that old-fashioned methods may suffice for many purposes and those who wish to retain them can often argue that no harm appears to have resulted from their use. One of the difficulties in all preventive medicine is that it is often impossible to trace any disasters that may occur if there is a breakdown in normal routine. On the other hand, there is certainly enough evidence to indicate that from time to time fatalities do occur as a result of breakdowns in sterilization. There is no refuting the argument that as far as surgical equipment is concerned it is our duty to see that those articles which really must be sterile are sterile when handed to the surgeon.

2 HOW SHOULD STERILIZATION BE DONE? Different types of equipment demand different methods of sterilization. For surgical dressings, packs, and many other fairly bulky items of equipment, steam under pressure is the most widely used and can be highly satisfactory. Here the problem is one of penetration of steam into materials from which it is important that the last trace of air should be removed. For this reason the pre-vacuum type of autoclave is very desirable. Autoclaves working by downward displacement of air can be effective and many which have not been working properly can be put into good working order (Howie and Timbury, 1956). Some of these if they have been manufactured to stand higher pressures and if they can satisfactorily pass insurance tests can be adapted for use as pre-vacuum sterilizers. But wherever new sterilizers are being installed it is desirable that pre-vacuum autoclaves should be specified. It has already been emphasized that these can be a good deal smaller than downward-displacement sterilizers intended to carry the same overall load (Medical Research Council Report 1960; Wells and Whitwell, 1960). The capital and running costs may therefore be much less than might be expected.

For emergency sterilization of instruments for use in theatre areas, there is no need for a high pre-vacuum sterilizer and a small downward-displacement type is useful. In these it is important that steam should enter very rapidly and air be removed quickly. Sterilizers of this sort, even though small, are best fitted with at least two discharge channels of adequate bore.

For the sterilization of bottled fluids, if it is not done on a commercial basis, a central sterile supply department needs a large downward-displacement sterilizer. The process is slow but devices are being

developed to give more rapid cooling and shorten the whole cycle of sterilization.

For syringes, the standard of sterilization at present is by dry heat either in the form of a hot air oven or a conveyor belt or other devices which are being developed. Autoclaves may be used for sterilization of syringes but they are not so reliable or effective unless the syringe is dismantled and reassembled after sterilization. This is in itself a disadvantage and for that reason alone dry heat is preferable. Other methods such as the aluminium block and perhaps the pressure cooker have advantages for the small-scale user.

In the future, it seems that irradiation may well be the most satisfactory method of sterilizing syringes, especially if these are produced industrially and pre-packaged. The same applies to rubber gloves. These can of course be sterilized effectively by steam under pressure but disposable rubber gloves sterilized by irradiation are likely to be the answer to many of the special problems created by rubber goods. Irradiation on an industrial scale may also be used increasingly for many other articles.

It seems that there will still remain for some time many articles which cannot be sterilized by any of these methods, and for this type of equipment ethylene oxide is likely to be the most appropriate. The probable scope of ethylene oxide in the future is discussed by Kelsey (1961).

3 WHERE SHOULD STERILIZATION BE DONE? This raises many problems of organization and administration. The whole subject is in such a fluid state at the present moment that what is said today may be untrue tomorrow. As we move from the old-fashioned boiler in the ward or in the theatre to a system of centralized sterilization in a hospital, the logical conclusion is to move still further in the direction of large-scale production of pre-packaged sterile articles on a commercial baisis at competitive prices. But the question how far this process should go and what functions central sterile supply departments will have depends on future developments in the costs of materials, packaging, and transport, the ease with which supplies can be distributed at economic rates, and the area over which this can be done.

4 HOW OFTEN SHOULD ARTICLES BE STERILIZED? One of the dangers of sterilization which is not always realized is the fact that an article once sterile may gradually become contaminated if it is stored for any length of time, and the time necessary for contamination to occur is quite unknown in many cases. Any competent bacteriologist, however great his faith in the filtering effect of the conventional cottonwool plug, is rightly suspicious of any sterile test-tubes with cottonwool plugs which have been lying about in his laboratory for weeks or months, especially if he has no means of being certain that the cottonwool plug has not at some time been removed and replaced. The extent to which contamination occurs in surgical dressings and other equipment depends on the wrapping and the container in which they are stored. This in itself is an important subject on which more work needs to be done.

5 WHAT IS MEANT BY STERILITY? This question is not so easy to answer as it seems at first sight. A few years ago most bacteriologists if asked what they meant by sterility would have been inclined to say that an object was either sterile or not. This is partly because when methods, such as sterilization by steam under pressure or adequate degrees of dry heat, are used there is usually such an enormous safety margin in the process that sterility can be guaranteed with almost complete certainty. On the other hand, with some of the newer methods of sterilization, such as ethylene oxide and perhaps irradiation, it is becoming obvious that the achievement of sterility is largely a statistical process and that we can set our standard at several different levels. With irradiation or ethylene oxide, for example, we can aim at a kill of 10^{10} organisms or 10^{7} or 10^{5}. It becomes therefore necessary to define sterility in terms of the attainment of a certain degree of killing and we have to make up our minds what sort of bacterial population we are likely to find in different types of material. There is little doubt that this argument also applies to steam though less obviously.

6 HOW DO WE KNOW WHEN AN ARTICLE IS STERILE? The whole question of proper sterility tests is complex. Practical and effective tests are not easy to devise. They fall into two main groups. In the first type we use a test organism whose resistance to the bactericidal agent is known and measure the number of organisms killed by certain doses or certain periods of exposure to the sterilizing agent. In the other type of test we simply take samples of the finished product and see whether or not they conform to certain accepted standards of sterility. The second kind of test is much more difficult to make reliable. In any laboratory there will always be a certain 'natural' contamination rate and it is not easy to decide, when a certain rate of contamination occurs, whether this represents the best that can be achieved with the existing technique even with articles which are theoretically sterile, or whether it represents a genuine breakdown in sterilization of the object under test.

With certain types of equipment the interpretation of sterility tests is made even more difficult by the nature of the wrapping in which the material has been sterilized. Pre-packaged syringes, for example, are assembled and then placed inside an envelope of some plastic material which is then suitably sealed. The syringes are sterilized and issued ready for use inside these containers or envelopes usually made of materials which have to be cut by scissors or in some similar way. The very process of removing the syringe from its container in order to perform a sterility test is liable to cause contamination. How serious this risk is is still uncertain but it is obvious commonsense that, having taken great care to see that equipment is sterile, we must also ensure that the container can be opened with the least possible risk of contamination.

7 HOW SHOULD STERILIZERS BE TESTED? There are three main ways in which we can be assured whether a sterilizer is functioning efficiently or not.

i The sterilizer itself must have adequate instrumentation so that we can see whether the basic conditions necessary for its proper functioning are being achieved, for example, in the case of steam, temperature and pressure gauges, in the case of ethylene oxide, proper measurement of humidity and concentration of gas, and in the case of irradiation, accurate control of dosage.

ii For determining whether the necessary conditions have actually been reached in the material undergoing sterilization various kinds of tell-tale indicator are essential. The best-known example of these is the Browne's tube, which, although not absolutely ideal, gives information of great value and can be used in the day-to-day control of steam or dry-heat sterilization procedures. Indicators of this kind have the great advantage that they can be put in various positions in the sterilizer inside the loads undergoing sterilization. By preliminary experiment it is possible to find out whether there are any areas in the sterilizer or any parts of a load which are likely to escape adequate sterilization but the great advantage of the Browne's tube is that it can be used routinely if not inside every load at least inside a sample number of loads as a routine. The essential feature of an indicator of this kind is that it should be small. A number of new indicators are being developed and it is to be hoped that these will soon be available on a commercial basis for all the main methods of sterilization. It is of course possible in the case of steam to measure temperature actually inside loads by means of thermocouples or resistance thermometers, provided that these are small and can be moved about from one position to another. But clearly a small tube, such as the Browne's tube, is much more useful for routine purposes provided it gives sufficiently reliable results.

iii Bacteriological tests can be carried out from time to time provided it is realized that they have some of the limitations referred to above and that sterility tests are not quite as easy to carry out as is sometimes thought. There is in fact considerable danger in occasional bacteriological tests being carried out in a hospital laboratory unless done by well-trained people who are well aware of the various pitfalls. Growth obtained from a test sample embedded inside a dressing pack may well indicate not a failure of sterilization at all but a failure in the technique employed in the laboratory. With some objects, especially if they are large, such failures are almost impossible to avoid, since it is extremely difficult to carry out an adequate sterility test on a large and bulky object without introducing some contamination in the process. On the other hand, the kind of sterility test which is often carried out in hospital laboratories can be even more dangerous in the opposite direction. For example, if proper controls are not used and if the organism used is too easily killed by heat, then the hospital pathologist may go on reporting sterile cultures till some disaster occurs. Only then does a thorough investigation reveal the fact that the sterilizer has not been functioning properly for a long time. In such cases the bacteriological tests are worse than useless as they give a false sense of security. Adequate inspection and control of the functioning of sterilizers are far more important than the purely routine multiplication of bacteriological tests which themselves may be quite uninformative. On the other hand, properly conducted bacteriological tests, preferably those in which test organisms of known resistance to the bactericidal agent are used, are the only direct method of telling whether the sterilizer is doing its job or not. The situation is very much the same as in the testing of water supplies. Bacteriological tests are the only tests which will tell us directly whether the bacterial content of a water supply conforms to certain standards but a single isolated test in inexpert hands is worse than useless. No bacteriological test is of any value at all, however expertly carried out, unless it is viewed in relation to the background of information about the source from which the water comes, the way in which it is treated, and the arrangements available for its distribution and maintenance of its freedom from contamination.

8 WHAT PART DOES STERILIZATION PLAY IN THE CONTROL OF HOSPITAL INFECTION? There is great concern at the present time about the increasing

frequency of staphylococcal and other infections in our hospitals (Williams, Blowers, Garrod, and Shooter, 1960). At the same time, as has been mentioned already, there have been many reports of breakdowns in sterilization and it is clear that failure to sterilize is widespread. It is natural that a connexion should have been suggested between hospital infection and breakdowns in sterilization. It has even been implied that if we could put our sterilization in order we should eliminate hospital infections or at any rate a large part of them. There is little to justify this attitude. Some infections which occur in hospitals are undoubtedly due to a breakdown in sterility: some cases of tetanus, for example, probably some cases of hepatitis, and an unknown number of other infections. But the staphylococcus which is causing most concern at the present time is quite easily killed by heat and there is not often enough evidence to suggest that a breakdown in sterilization is a major cause of staphylococcal outbreaks, though it may have been from time to time. Hospital infection spreads by many channels and efficient sterilization is only one of the ways by which its incidence may be reduced.

The true frequency of hospital infection is difficult to estimate but there is now general agreement that although the rate of post-operative sepsis is far lower than it was before the use of antibiotics, it is now a good deal higher than it was in the years immediately following their general introduction. Although in many ways surgical technique has made tremendous advances it is generally agreed that there is a real need for a tightening up of standards of operative and other techniques intended to reduce the risk of infection. One of these is the provision of sterile equipment. Here there is no doubt that standards have fallen to dangerously low levels. On these grounds alone it is essential that we should completely reorganize our sterilization procedures.

CONCLUSION

Two established methods of sterilization have stood up to the test of recent criticism, namely, the use of steam under pressure and dry heat. Other established methods such as boiling, steaming, and the use of liquid disinfectant are to be regarded no longer as methods of sterilization but rather as methods of disinfection. Of the newer methods, ethylene oxide and irradiation are the most promising. It is likely that for many years ethylene oxide will have a place for sterilizing certain special types of equipment, but though it is risky to prophesy it seems that irradiation is going to play the most useful part in the future. This inevitably means increasing centralization, increasing use of disposable equipment, and increasing industrialization of the whole process. We seem to be moving into an era in which sterilization will be much less a hospital responsibility but will be in fact an industrial process carried out by industrial methods and with adequate monitoring and methods of control on an industrial scale.

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Control of infection in hospital wards

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SYNOPSIS Some of the problems of ward management are reviewed. Methods suggested for dealing with them are probably not the ideals that should ultimately be attained but minimum standards to serve as immediate objectives. They concern indications for and methods of isolation, control of infection from staff, environmental contamination, and a few technical procedures. A new type of dressing towel for wounds is described.

Infection acquired in a hospital ward may directly cause disease; or it may create a symptomless carrier. Efforts to control infection must concern both of these processes, because though a symptomless carrier himself suffers no immediate harm, he remains a source from which others may be more seriously infected; moreover, he may re-infect himself if the organisms find their way from the site of carriage to some more susceptible tissue. The success of preventive measures must therefore be judged not only by their immediate effect on disease but also by their control of carrier rates for pathogenic organisms.

There are many ways by which bacteria may reach a patient, but none is necessarily more important than any of the others. So the value of many precautions that are now taken remains uncertain. The hope is sometimes expressed that with more detailed knowledge of the ways by which bacteria spread in a ward we may be able to concentrate on only a few but vitally important precautions, and to relax the many others that make our present system so tedious and so fallible. This hope will be fulfilled only if bacteria behave themselves: if all species and all strains spread in the same way at all times. There is no evidence that this is so and though some broad generalizations may be possible, categorical support or condemnation of any precaution cannot be based on its success or failure on a particular occasion. Assessment of the value of precautions against infection is difficult for yet another reason. Gillespie, Alder, Ayliffe, Bradbeer, and Wypkema (1959) found that several precautions, which by themselves had no discernible effect on infection rates, had a significant effect when applied together. Lack of effect from applying or withdrawing any one procedure must therefore be interpreted very cautiously. With these difficulties of interpretation and much conflicting evidence in mind, the physician or surgeon must choose the methods that he will apply in his own wards.

There are, perhaps, four basic principles of ward procedure for the control of infection: patients who are especially liable to spread infection and those who are especially susceptible to it should not be accommodated in the same ward; the ward staff should not themselves be dangerous sources or vectors of pathogenic organisms; the patients' inanimate environment should not become a reservoir of pathogens; and ward techniques must be those least liable to convey pathogens. For each of these principles, I make no attempt to define the ideals that are theoretically desirable. For the present it seems more useful to suggest the minimum standards we should try to reach. Nor can a review such as this cover all aspects of ward management. Among the many important problems not considered here are the pre-operative treatment of skin, methods for disposal of contaminated articles, and the proper use of antibiotics.

ISOLATION AND SEGREGATION

Here is a suggested list of infections for which patients should be removed from an open ward. They should also be moved when they are suffering from disorders making them particularly susceptible to infection, or when being treated with certain drugs which enhance susceptibility to infection.

1 THE ACUTE SPECIFIC FEVERS, *e.g.*, measles, mumps, chickenpox, whooping-cough, scarlet fever (and other streptococcal diseases), diphtheria.

2 OPEN TUBERCULOSIS.

3 INTESTINAL INFECTIONS: (*a*) *Shigella* dysentery; (*b*) *Salmonella* infections, *i.e.*, typhoid and paratyphoid fevers, food poisoning, symptomless carriers; (*c*) *E. coli* enteritis of infants and symptomless

carriers of locally pathogenic strains; (*d*) unexplained diarrhoea.

4 STAPHYLOCOCCAL DISEASE.

5 INCREASED SUSCEPTIBILITY TO INFECTION: (*a*) Treatment with steroid drugs; (*b*) acute leukaemia, especially during treatment with marrow-toxic drugs; (*c*) agranulocytosis; (*d*) uraemia; (*e*) burns and skin grafts.

Many of these indications are so obvious that no justification is needed here. Of the others, staphylococcal infections deserve special discussion.

It is remarkable that in many wards where elaborate precautions against staphylococcal infection are taken, little importance is attached to the apparently obvious one of removing patients with frank sepsis. Thus, patients who have been admitted with *Staphylococcus aureus* pneumonia, empyema, osteomyelitis, breast and other abscesses, urinary tract infection, and septic dermatitis are regularly treated in open wards alongside uninfected patients. Perhaps even more dangerous are those patients who became infected with staphylococci in hospital, because the organisms involved are more often epidemic strains and resistant to antibiotics; in this category are patients suffering from *Staph. aureus* enterocolitis, septic burns, post-operative wound sepsis, and the various forms of neonatal sepsis.

When patients must be removed from an open ward for any of these reasons more isolation accommodation than is usual must be provided, and until it is available, some form of priority must be devised. The very profuse discharge of organisms from patients with staphylococcal enterocolitis, pneumonia, septic dermatitis, and burns requires that they should be isolated. For patients who have been infected in hospital and from whom organisms are discharging profusely the need for isolation is still more imperative.

It is sometimes objected, however, that isolating septic patients cannot be expected to control infection while symptomless carriers of *Staph. aureus* remain in the ward. This is probably true during a major outbreak of sepsis caused by a highly virulent organism, when it may indeed be necessary to remove carriers of the epidemic strain, as well as the septic patients to break the chain of infection. In non-epidemic times, however, most of the symptomless carriers harbour strains that seem to lack virulence or a high degree of transmissibility, and these patients can safely remain in a well-managed ward. But the organisms in actual lesions have already proved their virulence and it seems unwise to give them an opportunity to prove their powers of spread.

METHODS OF ISOLATION For the acute specific fevers and intestinal infections the patient should be transferred to an infectious diseases hospital. For staphylococcal diseases needing surgical treatment this is rarely practicable though when it is, full use should be made of isolation hospitals, especially during a serious outbreak. The value of the various degrees of 'isolation' available in general hospitals has not been defined and careful bacteriological studies are very much needed.

Barrier nursing in an open ward depends on eliminating infection by direct and indirect contact and may actually do this if an exceptionally high standard of nursing is assured. It cannot, however, prevent airborne infection though it is not yet certain how great a part this plays in the spread of staphylococci in a ward. By studying the spread of *Staph. aureus* from known infant carriers and from one nurse, Wolinsky, Lipsitz, Mortimer, and Rammelkamp (1960) concluded that contact rather than airborne spread accounted for almost all the infections. But individuals vary greatly in their powers of aerial dissemination of staphylococci (Hare and Thomas, 1956; Hare and Ridley, 1958; Eichenwald, Kotsevalov, and Fasso, 1960), and studies of airborne spread will have to be made with subjects who are known to be profuse 'dispersers' before final conclusions can be reached on the importance of this method of studying spread of infection. For all this, there are many examples of failure to control the spread of *Staph. aureus* by barrier nursing, and, whether these are due to inadequacy of the system or failure to apply it properly, it cannot be recommended as a reliable method.

Isolation in a ward side-room is perhaps more often successful but failures are many if the patient is simply moved into it and attended in the usual way by nurses who also have to deal with uninfected patients in the main ward. Side-ward isolation should therefore always include the full ritual of barrier nursing as a safeguard against contact infection. Thus, all equipment needed for the patient is kept in the isolation room or is disinfected or destroyed as soon as it is brought out; ward staff wear gowns (which are kept in the isolation area) and masks when attending the patient and wash their hands before and after doing so.

Isolation rooms for septic patients should be ventilated by simple exhaust fans discharging to the outside so that air flows into them and not from them to the main ward. For patients who are being isolated for protection against infection, airflow in the opposite direction is theoretically needed but a reasonable compromise is to switch off the exhaust fan and to rely on natural ventilation from

an open window. The door of an isolation room should be kept closed.

Full isolation requires nurses who do not attend uninfected patients, and a separate room for each patient in a building set away from the general wards. Isolation units such as this are not part of the usual hospital design in Britain but they have sometimes been improvised during an emergency and, remaining in use afterwards, have shown their value by apparently limiting the spread of infection from occasional septic patients during non-epidemic times.

Only recently has the number of isolation rooms required for the control of infection become the subject of quantitative study. Results of current studies are not yet available but it seems likely that at least 30% of surgical and 15% of medical beds will be required. In most British hospitals such generous isolation facilities are not yet available and until they are, anything approaching full control of staphylococcal infection will be difficult.

Segregation During an outbreak of sepsis when there are more patients for isolation than there are rooms to put them in, an acceptable substitute for isolation is segregation, whereby infected patients are collected together in a special ward. This does not, of course, prevent them from re-infecting each other and it is a method that can only be accepted if there is no possibility of proper isolation. It is, however, infinitely preferable to leaving the septic and clean patients together in the same ward.

PREVENTION OF INFECTION FROM WARD STAFF

INFECTIOUS DISEASES Members of the hospital staff who remain on duty with even minor infections may be more dangerous than patients suffering from serious infective conditions. Nurses and doctors, perhaps from a mistaken sense of duty, often continue to attend patients while suffering from a sore throat, undiagnosed diarrhoea, purulent discharge from nose or ear, or staphylococcal sepsis such as generalized furunculosis, boils, styes, paronychia, and septic cuts or burns. Nurses are sometimes discouraged from reporting sick for these 'trivial' reasons and many doctors believe that their services are too valuable for them to hand over to a colleague for a few days. It is not easy to lay down general rules for behaviour of staff during minor illness or sepsis but the minimum requirements (apart from the obvious ones) should be:

Completely off duty for diarrhoea until proved bacteriologically safe, sore throat; one large discharging staphylococcal lesion; generalized furunculosis with several discharging lesions.

Not to assist at any aseptic procedure (e.g., wound inspection, wound dressing, or injection), with a discharging lesion on any part of the body; any septic lesion, whether discharging or not, on the hand or forearm.

SYMPTOMLESS CARRIERS The routine seeking and exclusion from duty of all carriers of pathogenic organisms amongst ward staff is impracticable and probably not necessary. As with the patients, however, it may be needed during an outbreak due to a clearly recognizable organism. Carriers of *Staph. aureus* are detected by swabbing the anterior nares. Phage typing should be used to identify the epidemic strain and antibiotic sensitivity patterns are sometimes useful for more rapid but partial identification. Clearance of the carrier state is often difficult but treatment that sometimes succeeds is the thrice-daily application of neomycin-chlorhexidine cream[1] to the noses of carriers for 10 days. In addition when washing and bathing they should regularly use toilet soap[2] or cream[3] containing hexachlorophene or tetrachlorsalicylanilide[4]. Persistent skin carriage is often controlled by the permanent use, both on and off duty, of these soaps even though carriage by the nose remains uncontrolled.

MECHANICAL CONVEYANCE OF ORGANISMS BY WARD STAFF Contamination of hands, and spread from them, can be reduced by applying these rules:

Use forceps or rubber gloves for all wound dressings and examinations and for handling drainage tubes, tracheal tubes, catheters, and other heavily contaminated objects.

Wash the hands before and after any procedure for which gloves, forceps, or sterilized equipment are used; after changing an infant's napkin or dressing the umbilicus; after rounds of temperature taking, bedmaking, or bedpans; after any item of service for a patient who is (or should be) isolated; after defaecation and before and after each spell of ward duty.

For all these purposes a 'surgical scrub' probably has no advantage over thoroughly washing the hands with soap and water and drying them on a clean towel. Conveyance of pathogens is reduced if the soap contains hexachlorophene (Lowbury and Lilly, 1960). Automatic roller-towel dispensers and good-quality paper towels are both satisfactory and should replace the communal towel. It may sometimes be preferable to use a sterile towel but when this is not done drying the hands on a clean but unsterilized towel is safer than leaving them wet.

[1] Naseptin cream (Imperial Chemical Industries Ltd.).
[2] Derl (Roberts Windsor Ltd.); Cidal (Bibby Ltd.).
[3] Phisohex (Bayer Products Ltd.).
[4] Breeze (Crosfields (C.W.G.) Ltd.).

Clothing The aprons and dresses of nurses attending infected patients, especially children who must often be lifted and carried, are sometimes heavily contaminated after only one day's wearing (Potter and Blowers, unpublished observations in a burns unit). Thus there seems some justification for a one-piece uniform, changed daily, instead of the usual daily clean apron and a dress that is worn for a week. Besides its possible bacteriological advantages, this means one less item of laundry each week than does the older system.

Cotton gowns offer only incomplete protection against contamination in either direction but they seem to be of some value (Cook, Parrish, and Shooter, 1958) so they should be worn over the uniform at least during wound dressings and during attention to patients in isolation. Impervious plastic gowns, disinfected by wiping with a hypochlorite solution containing 1% available chlorine[1], give better protection and seem preferable to cotton gowns for barrier nursing and isolation techniques.

CONTROL OF ENVIRONMENTAL CONTAMINATION

For many years, and especially in the last few, bacteriologists have been busy discovering inanimate reservoirs of pathogenic organisms in hospitals—walls, floors, ceilings, and furniture; bed-clothes, curtains, and crockery; and the air—while methods for disinfecting them have been increasing the cost and complexity of ward management. These activities should certainly not be discouraged, because the discovery of reservoirs and the invention of methods for disinfecting them are essential preliminaries to the full study of such organisms. It cannot be denied that disinfection of the whole environment is essential when there has been known heavy contamination with dangerous organisms. The need for frequent and energetic environmental decontamination at other times is less certain. Theoretically, it should reduce the risk of infection at any time, but whether or not it does so significantly has not been conclusively shown. Unfortunately, clear evidence on this point is not easy to obtain because, as has already been pointed out, the complexity of bacterial spread makes the evaluation of each pathway very difficult. Enthusiasts for particular methods will no doubt continue to apply them but it is hoped that they will accept responsibility for the steady, even though laborious, collection of evidence to support their views, rather than becoming tedious crusaders for unproven causes.

[1] For example, Chloros (Imperial Chemical Industries Ltd.) diluted 1 in 10, wiped on and left to dry.

My purpose here is not to enumerate all the disinfection procedures that might do some good, but to suggest, in the light of available evidence though without quoting it all in detail, minimum standards for environmental hygiene in hospital wards. Still higher standards may be desirable but those suggested are by no means universal and should serve as reasonable targets for the next few years.

WALLS, FLOORS, CEILINGS, AND FURNITURE The number of pathogens on these surfaces can be kept low by applying high standards of domestic cleanliness. This means that *floors* should be washed each morning, and dust and fluff should be taken up by vacuum cleaner after bed-making rounds. For routine purposes, mopping with very hot water, at not less than 70°C. (160°F.), and a synthetic detergent is sufficient. The routine use of disinfectants for washing floors gives little better results and most of those cheap enough for the purpose cause a persistent and unpleasant smell. Bacteria may multiply profusely in dirty mops and buckets of water if these are left for several hours. After each use the bucket and mop should therefore be rinsed with boiling water.

Broom-sweeping often raises as much dust as it collects, and slit-sampling shows an enormous increase in the number of airborne organisms while it is going on. This can be reduced by oiling the floors but the process is only really satisfactory for wood and not for modern non-absorbent floors. The best solution is to discard brooms and use vacuum cleaners. Built-in suction points have some advantages but are expensive to install and are less convenient than portable electric machines. Elsewhere in this issue, Dr. Bate discusses the types of machine that are safe for use in hospital (page 32).

Walls and ceilings cannot conveniently be cleaned as often as floors; nor does this seem necessary because dust collects on them more slowly and is less often disturbed from them than from floors. Perhaps a reasonable rule to follow here is that they should never be visibly dirty. The washing methods already described for floors should therefore be applied often enough to ensure this. It is a disturbing thought that there are still hospitals where even this standard is not reached.

Ledges, furniture, and beds should be dusted daily with a damp cloth.

These are the routine methods. If, however, an isolation room has been occupied by a patient suffering from infectious disease (and this includes staphylococcal sepsis) specific decontamination is needed. The whole room may be treated by sealing it, spraying water to increase humidity, then

generating formaldehyde and leaving the room closed for six hours. For each 1,000 cu. ft. of room space, 5 oz. (150 g.) potassium permanganate is added to 10 oz. (280 ml.) of formalin. Sulphur dioxide is ineffective. A satisfactory and often more convenient method is to wash all surfaces, furniture, and the bed with hypochlorite solution (see footnote on page 21) to which has been added some household detergent.

If a septic patient has been barrier-nursed in an open ward, it may suffice to wipe over the bed and bedside furniture in this way. But if a large ward has been closed because of widespread sepsis it should not be re-opened until the whole room has been disinfected by one of these methods.

SCREENS AND CURTAINS Separate pull-round curtains for each bed in an open ward harbour more dust than a few sets of portable screens. Their social advantages are so great, however, that it seems unjustifiable to condemn them on theoretical and unevaluated bacteriological grounds. It is arbitrarily suggested that the curtains be removed and disinfected each month, when the overhead rails and runners should also be damp-dusted. Cotton curtains should be of colour-fast material so that they may be laundered, and thus disinfected by boiling. Plastic materials have the theoretical advantage that they can be wiped with a disinfectant *in situ* or in a ward annexe; but this occupies more of the ward staff's time than removing cotton curtains and sending them to the laundry, so is often postponed for long periods. Thompson and Webb (1960) describe fibre-glass curtains which can be washed at high temperature in the laundry or may be dipped in a disinfectant in the ward annexes and re-hung almost immediately.

BEDDING There is no doubt that bed-clothes can become heavily contaminated with pathogenic organisms from a patient's lesions or from sites of symptomless carriage. Sheets are adequately disinfected by the boiling they normally get during laundering; but hospital blankets are rarely washed, and then only by a low-temperature process which neither removes nor destroys bacteria. It is argued that contaminated blankets, carrying organisms from a long succession of patients in each bed, may directly infect a new occupant, that organisms scattered from them into the air during bed making may help to maintain high nasal carrier rates of staphylococci derived from septic lesions; and that hospital beds should therefore be equipped with disinfected blankets for each new occupant. The theoretical advantages of this are clear, but there are conflicting reports of its actual value. These are perhaps summarized by the experiences of Gillespie *et al.* (1959) and Gillespie, Simpson, and Tozer (1958) who found that regular blanket disinfection as the only new precaution had no detectable influence on *Staph. aureus* sepsis or carrier rates, but when applied with other precautions appeared to play a part in their control.

Whether or not this fairly expensive routine is wholly justified on bacteriological grounds, apart from its obvious aesthetic desirability, remains to be shown. In the meantime, the following routine is suggested as a minimum standard:

(1) Sheets should be laundered at least weekly and whenever a bed is vacated.

(2) Blankets, mattresses, and pillows should be disinfected (*a*) every three months; (*b*) whenever visibly soiled; (*c*) after use by any infected patient who has been isolated or should have been isolated according to the indications already given; (*d*) after use by every patient during an outbreak that has resisted the routine precautions; (*e*) before re-opening a ward that has been closed because of infection.

Woollen blankets may be disinfected by washing them at the usual low temperature and using a detergent combined with a quaternary ammonium or other suitable disinfectant substance[1]. It was once hoped that incorporation of the disinfectant in the last rinse instead of the washing stage would confer self-disinfecting properties on the blankets. But though fibres from blankets so treated cause zones of inhibition on a culture plate seeded with staphylococci, there is no appreciable killing of organisms in the blanket itself during use, presumably because the disinfectant does not act when dry. Woollen blankets may also be disinfected by high-temperature washing provided this is just below boiling point (British Launderers' Research Association and International Wool Secretariat, 1959). This causes no more damage to the blankets than the low-temperature wash but woollen blankets will stand only about 60 washes in any case, so disinfecting them regularly must always be fairly expensive. Cotton cellular or terry-towelling blankets are easily disinfected by washing at boiling point or just below and will stand several hundred such treatments (Blowers, Potter, and Wallace, 1957). For pillows and mattresses enclosed in plastic covers, it is usually sufficient to wipe the covers with hypochlorite solution.

CROCKERY This should be washed, or rinsed after washing, in water at 60°C. (140°F.).

[1] For example, Vantropol BQ (Imperial Chemical Industries Ltd.); Hytox (Domestos Ltd.); Steravol (Laporte Ltd.); Comprox-panacide (B.P. Detergents Ltd.).

NURSERY EQUIPMENT

The procedures already given are obviously applicable in nurseries but three pieces of equipment on which many babies are successively placed each day need special attention. Nursery baths (and indeed baths in all wards) should be disinfected after each use by the effective method of Boycott (1956). One to two gallons of very hot water are run into the bath; ½ fl. oz. of undiluted hypochlorite solution and enough domestic detergent for effective cleaning is added to it and the bath is thoroughly mopped with this mixture. The pans of the baby scales should be wiped after each use with a non-irritant disinfectant such as chlorhexidine[1]. This can be properly done only if metal or plastic pans replace the widely-used wicker ones. The tables on which babies are placed for changing their napkins or other attention should be similarly treated and covered by a freshly laundered towel for each baby. But the risk of cross-infection is reduced if as many as possible of these tasks are done with the babies in their own cots.

One other procedure of vital importance in the prevention of nursery epidemics is the preparation and bottling of feeds for the babies. This should be done by a nurse who does not handle the babies and in a department set aside and equipped for the purpose. Suitable equipment and recommended methods cannot be fully discussed here and the reader is referred to the detailed recommendations of Perkins (1956).

THE AIR There is yet no conclusive evidence of the importance of airborne infection in wards. Direct infection by this route of properly covered surgical wounds seems unlikely, but staphylococcal infection of the respiratory tract causing pneumonia or symptomless nasal carriage seem real possibilities. Plenum ventilation with a high exchange rate as used in operation theatres would reduce the number of airborne organisms but the cost of applying it to a whole hospital would be enormous. Recirculation ventilation of wards, with filtration to remove bacteria, is less expensive but its effect on infection is not known. Recirculation systems without filtration and serving a whole hospital may actually assist the spread of infection between wards.

Hudson, Sanger, and Sproul (1959) suggest treating the air and entire ward contents with a long-acting disinfectant, bis (n-tributyl) tin oxide. This reduces the number of airborne organisms by about four-fifths but the effect on infection is not yet known.

[1] Hibitane (Imperial Chemical Industries Ltd.); 0·5% alcoholic solution or the 1% obstetric cream are both effective for scale pans.

The most recently proposed method (Elek and Fleming, 1960) is to spray the ward with a new synthetic penicillinase-resistant penicillin. This must surely remain experimental until the properties of the drug have been more fully studied. Before it can be contemplated as a routine measure, much more must be known about its effect in creating or selecting resistant strains, in leaving the way open for serious Gram-negative and *Candida* infections, and in causing human sensitivity. At present, therefore, control of airborne infection in wards must still depend on preventing dissemination of organisms by isolation and by other techniques already recommended.

SPECIAL TECHNIQUES

Of the many special ward techniques that give opportunity for infection, only two will be discussed here: wound dressings and continuous drainage methods.

WOUND DRESSINGS Techniques for wound dressings must allow no opportunity for infection to or from the wound by direct or indirect contact. Reliable methods for a dresser and assistant or for an unassisted dresser are described in a Ministry of Health report (1959) so are not given in detail here. The number of ward sisters and sister tutors who have neither seen nor heard of this publication is remarkable. Because the importance of training nurses in the control of infection is so great this deficiency should be put right.

The setting of dressing trolleys is simplified and thus made safer by using individual dressing packs, disposable gallipots, and disposable paper bags for dirty dressings.

One further improvement in dressing technique is now available, namely, the use of water-repellent dressing towels. The usual dressing towels are water-absorbent so that wet instruments placed on them may be contaminated when the water soaks through to an unsterile table top beneath (Fig. 1). It is sometimes necessary to put a towel over the bedclothes around the wound and though instruments should not be placed on this, they sometimes are, when the risk of contamination is even greater. Some types of disposable paper dressing towels are even more absorbent than cotton so may increase rather than reduce, as is claimed, the risk of infection. Cotton fabrics of the Ventile[1] type do not allow water to soak through it unless it is very hot. These materials are being successfully used for the sleeves of surgeons' gowns to prevent skin organisms passing through to the wound when the sleeves are wetted,

[1] Messrs. John Southworth & Sons Ltd., Clitheroe, Lancashire.

FIG. 1. *Wet forceps on an ordinary cotton dressing towel. The water soaks through to the underlying surface, from which the instrument may then be contaminated.*

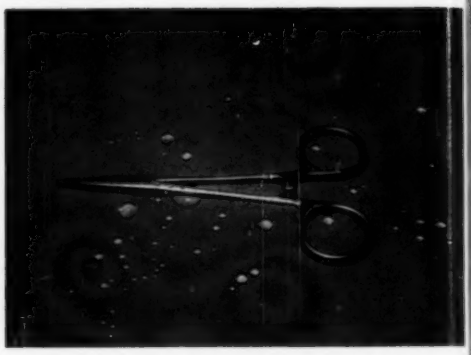

FIG. 2. *Wet forceps on a Ventile cotton dressing towel. Water remains as globules which neither wet the surface nor pass through the material.*

as they often are during an operation. A more recent application is to use Ventile fabrics for dressing towels to reduce the risk of contamination from underlying surfaces. Drops of water remaining on a Ventile dressing-towel without soaking through are shown in Fig. 2.

Wound Dressing Rooms The value of specially ventilated wound dressing rooms has been clearly shown by Bourdillon and Colebrook (1946) and by Lowbury (1954). For patients in open wards all wounds, whether infected or clean, should therefore be done in a specially equipped and ventilated dressing-room. For patients already in isolation, however, the dressing should be done in the isolation room unless it is so complicated that the special facilities of the dressing-room are essential.

A dressing-room that is used for septic work soon becomes a concentration area for pathogenic organisms unless it is thoroughly cleaned after every session of dressings and unless it is specially ventilated. Positive-pressure ventilation, as recommended by Bourdillon and Colebrook, prevents contamination of clean wounds with airborne organisms from the wards. For a dressing-room that is often used for septic wounds and closely communicates with a ward, however, this may cause heavy contamination of the ward so a balanced ventilation system with input and extraction fans of equal capacity is preferable. A rapid turnover of air is needed and for rooms of up to 2,000 cu. ft., a ventilation rate of 650 cu. ft. per minute will clear heavy aerial contamination in about 10 minutes. It is rarely possible to equip dressing-rooms with the complicated and expensive ventilation plant of the type used in operating theatres. A simple and inexpensive plant suitable for dressing-rooms is described by Williams, Blowers, Garrod, and Shooter (1960).

CONTINUOUS SUCTION APPARATUS Surgery of the urinary tract is complicated by post-operative infection perhaps more often than any other type of surgery. Most of this infection depends on common but avoidable faults of indwelling catheter drainage systems. Pyrah, Goldie, Parsons, and Raper (1955) and Miller, Gillespie, Linton, Slade, and Mitchell (1958, 1960) show that post-operative urinary sepsis is all but eliminated by using closed drainage apparatus. In many wards, any urine drainage system using a bunged bottle is accepted as a closed one but this is far from being so. Miller and his colleagues showed that even temporary disconnexion of the system for bladder irrigation or for some other reason allowed infection to occur as often as did an open drainage system. Their apparatus, which is strongly recommended, includes an irrigation attachment that can be used without breaking the closed system. Failure to sterilize an already infected bladder is often due to its constant reinfection by organisms that ascend from the accumulated urine in the bottle. This is prevented by putting 100 ml. of formalin in the bottle.

Drainage from the thoracic cavity, too, often allows infection unless a closed system is used. For this, however, a disinfectant cannot be put in the bottle because it may be aspirated into the chest during coughing. Contamination of the fluid should be prevented by a cottonwool plug in the air outlet tube and by changing the bottles with the aseptic care of a surgical operation.

I am grateful to Dr. Stanley Wray and Mr. William Bound for their help with the photographs.

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Chemical disinfection in the prevention of infection in hospital

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SYNOPSIS Much cross-infection can be prevented by using chemical disinfectants to reduce the numbers of pathogens in sources such as superficial lesions and carrier sites, and by interrupting routes of spread. Examples are given to show the value of using disinfectants in the light of epidemiological knowledge.

When aseptic surgery superseded the antiseptic methods of Lister, its advantages were so obvious that its shortcomings were sometimes overlooked. It was often assumed, quite wrongly, that complete sterility could be achieved at the operation site by physical and mechanical methods of excluding and destroying bacteria. Disinfectants and antiseptics (that is, disinfectants which can be safely applied to tissues) continued in use. But on the whole they were used empirically and with insufficient understanding of their limitations. This was perhaps the inevitable result of difficulties inherent in the realistic evaluation of disinfectants. Laboratory tests of their activity *in vitro* often gave little guidance as to their clinical value. The resulting uncertainties in choosing between the different agents available and in deciding what concentrations to employ have caused much confusion.

Recent years have brought a lively realization of the limitations of the aseptic methods as practised in operating theatres. Some defects have resulted from faulty methods of heat sterilization. But correction of such faults, although important, cannot provide sterile operating fields. Even if instruments, dressings, and fabrics are sterile, bacteria may reach susceptible tissues in several ways, for example, from the air, from the patient's skin or mucous membrane at the site of operation, and from the surgeon's skin through holes in his gloves or through the sleeve of his gown if it becomes wet. Sources and vectors of infection such as these cannot be completely sterilized; but the amount of contamination (except for the air) may be greatly reduced by means of disinfectants.

In the wards and labour rooms, as in the theatre, prevention of infection should be based primarily on physical and hygienic measures, such as the provision of heat-sterilized instruments and dressings, the use of non-touch techniques, and the avoidance of overcrowding. Disinfectants and antiseptics are not substitutes for good hygiene but should supplement it. Experience has shown, however, that the supplementation may be very valuable, and may substantially reduce cross-infection rates which, even in well-conducted wards, are sometimes disturbingly high.

In the control of hospital infection, therefore, chemical disinfection plays an important, though incomplete, role. Here it is appropriate to recall the distinguished pioneer work of Leonard Colebrook on the epidemiology and prevention of streptococcal puerperal infection and of infection in burns (Colebrook, 1950; 1954). Colebrook's work provided valuable patterns for subsequent investigations in other fields by demonstrating the importance of a thorough understanding of the epidemiological behaviour of the pathogens, and the value of combining physical methods, antiseptics, and systemic chemotherapy for the control of infection.

This paper describes some applications of chemical disinfection to the prevention of infection in general and maternity hospitals. It does not contain a comprehensive account of disinfectants and their uses. For this, reference should be made to textbooks such as those by Sykes (1958) and Williams, Blowers, Garrod, and Shooter (1960).

In theory, cross-infection might be prevented by interrupting the routes of spread of the pathogens, or by eliminating their sources. In practice, a combined attack usually succeeds best. The ability of a pathogen to cause infection depends on its numbers as well as on its virulence. Hence, even when several vectors of infection can be recognized and treated, it is wise to pay attention also to the sources, in order to reduce the load of infection as much as possible. It may be practicable to remove the sources by

isolating all patients with open infections. But in obstetric and surgical units, full isolation may be impracticable; and in any case, many sources cannot be recognized without elaborate bacteriological study. In these circumstances, much can be achieved by the chemoprophylactic treatment of potential sources (superficial open lesions and carrier sites) in order to protect them from colonization by the pathogen, or at any rate restrict its multiplication. It is usually easier to exclude the pathogen from a potential source than to eliminate it from a site already infected by it. The effectiveness of chemoprophylaxis varies greatly in different circumstances. Its greatest successes have been achieved in burns units and against staphylococci in maternity hospital nurseries, where the potential sources are very superficial and accessible to topical antiseptics.

Programmes for the prevention of cross-infection preferably should be selective, with emphasis on measures against demonstrably important sources and vectors. Thus, for the control of staphylococcal infection in surgical wards, it is more important to disinfect the baths, the bedding, and the nurses' hands than the walls and floor; and in maternity units, environmental hygiene will be largely wasted unless the staphylococci are prevented from multiplying freely in the infants' carrier sites. Nevertheless, a bactericidal system which would destroy pathogens in all parts of the hospital environment, including the air, might have much to commend it. The system described by Hudson, Sanger, and Sproul (1959) uses this approach. Complex mixtures of chemicals (for particulars of which reference should be made to the original article) were used regularly to disinfect a wide variety of fomites, including floors, baths, bedding, etc. Air was treated by re-circulating it through disinfectant-treated filters. Substantial reductions in the numbers of airborne bacteria were observed. Preliminary experience at the Hammersmith Hospital has confirmed these findings, but further observations will be needed to determine the effect on cross-infection (Mary Barber, personal communication).

Chemicals with selective antibacterial activities, although poor general disinfectants, may be useful for controlling particular infections. Thus, hexachlorophene and quaternary ammonium compounds are valuable for prophylaxis of staphylococcal infection but not of infection by *Pseudomonas pyocyanea*. Antibiotics such as neomycin and bacitracin, which are rarely administered systemically, may be applied as topical antiseptics. They should be used in combinations, or mixed with synthetic agents such as chlorhexidine, in order to minimize the risk of producing resistant strains.

DISINFECTANTS AND THE PREVENTION OF PARTICULAR INFECTIONS

HAEMOLYTIC STREPTOCOCCAL INFECTIONS IN OBSTETRICS. Reference has already been made to the pioneer work of Leonard Colebrook. The declining incidence of peurperal sepsis, following the introduction of sulphonamides and penicillin, has not lessened the importance of asepsis and antisepsis in midwifery. The demonstration by Colebrook and Maxted (1933) that chloroxylenol was actively and persistently lethal to streptococci in concentrations which were harmless to tissues showed the way to chemoprophylactic procedures in other fields and against other organisms. Newer disinfectants, such as chlorhexidine, might be preferable to chloroxylenol for obstetrical use; but the infrequency of streptococcal infections nowadays makes it difficult to compare different disinfectants.

INFECTION OF BURNS Burns very readily become colonized or infected by a variety of organisms, of which the most important is *Streptococcus pyogenes*. *Staphylococcus aureus*, coliform bacilli, and *Ps. pyocyanea* are also common, but their pathogenic roles are less firmly established. Methods of reducing infection have been intensively studied in work initiated by Leonard Colebrook and his colleagues in Glasgow (Colebrook, Gibson, Todd, Clark, Brown, and Anderson, 1944) and later in Birmingham. Their aim was not to achieve sterility but to exclude the more important pathogens, particularly *Str. pyogenes*. Their methods were mainly physical (plenum ventilation and 'non-touch' techniques) but were supplemented by chemoprophylaxis with penicillin cream applied topically. Lowbury and his colleagues in Birmingham have continued to evaluate these and other techniques, in an important series of controlled trials (Lowbury, 1960). Topical applications of antibacterial agents were shown to have considerable value. The incidence of streptococcal infections was greatly diminished by prophylaxis with penicillin cream (1,000 unit/g.). Infection by *Ps. pyocyanea* and coliform bacilli was diminished to a lesser, but still significant degree, by means of polymyxin cream (1 mg./g.). Staphylococcal infections were often prevented by creams containing neomycin (2 mg./g.) and chlorhexidine (1 mg./g.). A cream containing polymyxin, neomycin, and chlorhexidine was effective in prophylaxis against all the organisms; many burns remained apparently sterile, and added infection by *Staph. aureus*, in particular, was greatly reduced.

The work of Lowbury and his colleagues also threw light on the pathogenic roles of the bacteria which commonly colonize burns. The exclusion of

Str. pyogenes greatly increased the percentage of successes with skin grafting (Lowbury and Cason, 1954). Similar improvements in healing were observed when burns were protected from *Ps. pyocyanea* and *Staph. aureus*, thus providing evidence that these species also are harmful to burns, and should not be dismissed as saprophytes (Jackson, Lowbury, and Topley, 1951; Lowbury, 1957).

STAPHYLOCOCCAL INFECTION IN MATERNITY HOSPITALS

The part played by staphylococcal sources can be clearly observed in maternity hospital nurseries. The organisms are able to multiply rapidly, chiefly in the carrier sites of the infants themselves (Cunliffe, 1949; Gillespie, Simpson and Tozer, 1958; Hurst, 1960), and so produce an enormous staphylococcal 'pool' from which infection is readily spread to other infants and to adults. Keeping the infants in separate rooms with their mothers may lessen the danger though it does not remove it, so long as the infants are handled by a common nursing staff. Gillespie *et al.* (1958) found that the principal carrier sites were the umbilicus, nose, groin, and perineum, and less often, other flexures. Fortunately, all these sites are readily accessible to antiseptics. Farquharson, Penny, Edwards, and Barr (1952) and Baldwin, Rheins, Sylvester, and Shaffer (1957) reduced cross-infection by bathing the infants in solutions of hexachlorophene. In Bristol, as in many other centres, daily bathing of infants is not practised, and it was therefore found more convenient (and at least as effective) to apply hexachlorophene in a dusting powder. The infants are cleaned with sterile cotton wool and saline soon after birth. The cord remnant is trimmed short, and sealed with a plastic material (Octaflex), and a pre-sterilized talcum powder containing hexachlorophene 0·3% (Ster-Zac) is freely applied to the umbilical area, groins, perineum, axillae, and trunk, before the infant goes to the nursery. Thereafter the dusting is repeated every time the napkin is changed. In controlled trials, this treatment was found to reduce staphylococcal carriage by umbilicus and skin to small fractions of their previous levels, and to halve the incidence of nasal carriage. The incidence of skin sepsis among the infants was reduced from about 6% to less than 1% (Simpson, Tozer, and Gillespie, 1960), and there was a corresponding reduction in the incidence of maternal breast abscesses (Corner, Crowther, and Eades, 1960).

In recent trials (to be reported separately), the anterior nares of the powdered infants were treated once a day with a water-miscible cream (Naseptin) containing neomycin sulphate (0·5%) and chlorhexidine hydrochloride (0·1%). There was a further slight reduction in the infants' sepsis rate, but since this was already low, the improvement was necessarily small. Further experience will be needed to determine the clinical value of prophylactic nasal cream used in addition to hexachlorophene powder. The two measures together greatly reduced the numbers of 'hospital' staphylococci carried by the infants and mothers into the outside population (Donnison, Gillespie, Simpson, and Tozer, 1960).

Hexachlorophene powder is now used routinely in all maternity nurseries in Bristol. Several thousands of infants have been treated, with no skin irritation or other untoward effect. A powder containing chlorhexidine 1% was equally effective in preventing cross-infection, but caused some delay in the separation of the umbilical cord stump (unpublished observations).

The success obtained from treating sources was in striking contrast to the disappointing results of treating vectors. However, a controlled trial showed that the disinfection of nurses' hands with 1% chlorhexidine cream caused a small but significant reduction of cross-infection among the infants (Gillespie *et al.*, 1958).

URINARY INFECTION IN UROLOGY AND GYNAECOLOGY

Urinary infection often follows prostatectomy and continuous drainage of the bladder by indwelling catheter (Dukes, 1928; Durham, Shooter, and Curwen, 1954; Gillespie, 1956; Gillespie, Linton, Miller, and Slade, 1960). Kass (1957) found that even a single catheterization carried a 2-4% risk of infection. The causative organisms sometimes come from the patient himself, and sometimes from others, by a variety of routes. But except when an additional portal of entry has been artificially created, *e.g.*, by suprapubic bladder drainage, there is only one important final route of infection, the urethra and the indwelling catheter lying in it.

To prevent infection, it is necessary to destroy any pathogens which may have entered the urethra before instrumentation, and to exclude them during and after operation. Alternatively, bacteria which have entered the bladder may be washed out again with antiseptics. These principles may be applied as follows:

Prostatectomy The urethra may be disinfected by instilling 5-10 ml. of 0·05% chlorhexidine in glycerine, as soon as the patient is anaesthetized and causing it to be retained for about 10 minutes. The same solution may be used to lubricate urethral instruments. Infection transferred on cystoscopes from other patients may be avoided by ensuring that the instruments are thoroughly disinfected, after rinsing with water, by vertical immersion in an aqueous solution of chlorhexidine 1/5,000 with mercuric oxycyanide 1/2,000 for 15 minutes, or in 0·2%

chlorhexidine in 70% spirit for two minutes. Alternatively, the instruments may be disinfected by 'pasteurization' as described by Francis (1959). Retrograde infection *via* the indwelling catheter can usually be prevented by draining and irrigating the bladder by means of a closed apparatus in which sterility is maintained by formaldehyde (Miller, Gillespie, Linton, Slade, and Mitchell, 1958). The adoption of all these measures brought about a reduction of urinary infection after retropubic and transurethral prostatectomy from 83% to 6% (Gillespie *et al.*, 1960).

Simple Bladder Drainage With open drainage, infection is almost inevitable. The lower end of the drainage tube becomes heavily contaminated by contact with urine in the collecting vessel, and bacteria are readily carried up by bubbles which ascend in the tube when the patient moves. It is therefore important to keep the collecting urine sterile, and desirable also to exclude air bubbles. In the method described by Miller, Linton, Gillespie, Slade, and Mitchell (1960), these purposes are achieved by keeping the end of the drainage tube below the surface of *liq. formaldehyde* B.P.C. The apparatus is simpler than that used for prostatectomy patients since there is no need to wash out blood clots. The collecting bottle must be kept below the level of the bladder so as to avoid the risk of siphoning formalin back. A narrow-bore plastic catheter should be used to minimize urethritis (Gibbon, 1958). The use of this method for draining the bladders of patients with acute retention of urine reduced the incidence of infection from 73% to 10% (Miller *et al.*, 1960).

Kass and Sossen (1959) adopted a different approach to the problem of preventing infection with continuous drainage. They irrigated the bladder continuously with 0·25% acetic acid, in order to destroy, or wash out, any organisms which had entered it. The rate of irrigation was controlled by means of an electrical device. The infection rate was reduced from 100% to 10%.

Gynaecological Operations The bladder usually has to be emptied artificially for some time after a colporrhaphy operation. This is sometimes carried out by inserting an indwelling catheter for the first few post-operative days, followed if necessary by intermittent catheterization for another day or so, until normal micturition is completely restored. Alternatively, the bladder may be intermittently catheterized from the start. In one hospital, where the former procedure was used with open drainage, 98% of the patients acquired urinary infections. The incidence was reduced to 76% by substituting closed for open drainage; most of the patients now became infected during the subsequent intermittent catheterization (Gillespie *et al.*, 1960). The main sources of infection were considered to be the contaminated open drainage apparatus, and the patient's own urethra.

Paterson, Barr, and Macdonald (1960) also reported a very high incidence of infection (92·5%) in patients treated by indwelling catheter (presumably with open drainage). By changing to intermittent catheterization, the incidence of infection was reduced to 70%. Paterson and his colleagues then tried the effect of instilling 2 oz. of 1 in 5,000 (w/v) aqueous solution of chlorhexidine diacetate into the bladder after every catheterization. This procedure brought about a notable improvement, with a fall in the infection rate to 13·5%.

It may not always be desirable to instil disinfectant into the bladder. It might also be possible to protect the patient from infection by disinfecting the urethra before passing the catheter. This method is now being tested in Bristol. A solution of chlorhexidine digluconate 0·5% (w/v) is made up in a mixture of polyethylene glycols and water, which is semi-solid at room temperature but quickly liquefies at body temperature (G. H. Darling, personal communication). A small quantity is introduced into the urethra about a minute before catheterization, by means of a special syringe. Preliminary results with this method have been encouraging.

Urine as a Reservoir of Infection An open drainage apparatus may be an important reservoir of cross-infection. Gram-negative bacilli and other organisms multiply in the collecting vessels and contaminate the hands of the nurses who empty them. The introduction of a closed drainage apparatus containing formaldehyde was followed by the disappearance of several antibiotic-resistant strains from the wards in which they had previously been endemic (Gillespie *et al.*, 1960). Urine bottles and bedpans may also constitute reservoirs of infection in urological wards. McLeod (1958) pointed out that nurses' hands may be contaminated when rinsing and emptying urine bottles; urine should therefore be disinfected before emptying the bottles, which should then be terminally disinfected.

Urine containing staphylococci is especially dangerous in surgical wards. The procedure adopted to minimize this danger in a ward in the Bristol Royal Infirmary (with several urological beds) is to add 1 ml. of Tego 51 to each bottle before use. (For information about Tego compounds, which were developed in Germany, see Frisby, 1959.) This method is not entirely satisfactory, however, especially when the urine contains a large amount of pus. Other methods should be investigated in order to find a reliable, cheap, safe, and convenient procedure.

STAPHYLOCOCCAL INFECTION IN SURGICAL WARDS

The chief sources of infection are the noses of patients who become carriers, and the open lesions (*e.g.*, open and drained wounds, bedsores, etc.) which readily become colonized by staphylococci. Urine, sputum, and furuncles and infected closed wounds which discharge staphylococcal pus are also important, though usually less numerous than the first-mentioned sources. Many of the lesions are not superficial enough to be easily protected by local chemoprophylaxis. Hence, staphylococcal cross-infection is more difficult to control in surgical wards than in maternity units. Nevertheless, a combination of disinfectant measures directed against important potential sources and vectors can greatly reduce the amount of cross-infection. In prolonged controlled trials in Bristol, staphylococcal infections were reduced to about one-third of their former frequency in a ward where several precautions were simultaneously applied (Gillespie, Alder, Ayliffe, Bradbeer, and Wypkema, 1959). Subsequent experience in two more wards was similar, and showed that when the load of infection has been reduced, some precautions may be discontinued, at any rate temporarily, without the cross-infection rate returning to its former value (results to be published separately).

The system now used in the Bristol Royal Infirmary is to apply all the precautions together for several weeks, and later to withdraw one measure (the Polybactrin spray). The precautions are as follows:

AGAINST SOURCES 1. For nasal prophylaxis, neomycin-chlorhexidine cream (see above) is applied twice daily to the anterior nares of all patients from the day of admission. The cream is smeared inside each nostril with the finger tip, and the nostrils then massaged gently from outside. Most patients can apply the cream themselves, but must be supervised by a nurse as part of a regular ward routine. The nurse applies the cream to patients who are unable to do so themselves, by means of glass rods or swab sticks.

This method has been used for three years in two 30-bed wards and for one year in another, without producing neomycin-resistant staphylococci.

2. Initially, all superficial open lesions are sprayed with a mixture of neomycin, bacitracin, and polymyxin (Polybactrin, Calmic Ltd.) at every dressing. The inner layer of gauze is also sprayed. A controlled trial, to be reported separately, showed that this treatment protected a significant proportion of the lesions from staphylococcal colonization. Because it is expensive, the use of Polybactrin is stopped after some weeks, provided that routine swabs from patients' noses and open wounds show a marked reduction in staphylococcal cross-infection. Thereafter the lesions are treated instead with a sterile powder of calcium phosphate containing chlorhexidine dichloride 1% (w/w). The value of this measure is so far unproven. As an additional safeguard the dressings may be impregnated with chlorhexidine before autoclaving (V. G. Alder, personal communication).

3. Urine of urological patients is disinfected before emptying the urine bottles (see above). Special care is also taken to dispose safely of sputum and faeces which are heavily infected with staphylococci.

AGAINST VECTORS 1. Bedclothes, including blankets, bedcovers, and dressing gowns, are disinfected at intervals of not more than 14 days, or whenever the patient leaves hospital. High-temperature laundering is sufficient to disinfect cotton blankets and other non-woollen goods. Woollen blankets also may be disinfected in hot water, if rendered slightly acid (British Launderers' Research Association and the International Wool Secretariat, 1959), or by means of steam at subatmospheric pressure (V. G. Alder and W. A. Gillespie, to be published). If chemical methods are preferred, the choice lies between using a quaternary ammonium disinfectant during laundering, preferably by a one-stage method (*e.g.*, Hytox process), and a gaseous disinfectant. Warm, moist formaldehyde vapour is reasonably effective if penetration is assisted by a partial vacuum (Gillespie and Alder, 1957). Best results are obtained by using an efficient pump to provide a high vacuum. Ethylene oxide has also been used to disinfect blankets (Freeman and Barwell, 1960).

2. Crockery is disinfected by heat or by means of a detergent mixture containing a quaternary ammonium compound (*e.g.*, Vantropol, B.Q.).

3. The ward barber's brushes and razors are disinfected in 70% spirit for 10 minutes after use. A special trolley with a jar of spirit and sufficient brushes must be provided.

4. Nurses and other staff wash their hands with hexachlorophene toilet soap, dry with paper towels, and rub in Hibitane hand cream (chlorhexidine 1%).

5. Patients' baths are disinfected by adding hexachlorophene to the actual bath water (Ayliffe, Alder, and Gillespie, 1959). The water used for 'blanket bathing' bed-ridden patients is similarly treated. This measure, and the next, also have a disinfectant action on the skin.

6. The buttocks, back, and perineum of bed-ridden patients are dusted with hexachlorophene talcum powder (Ster-Zac).

STAPHYLOCOCCAL INFECTION IN OPERATING THEATRES

The control of staphylococcal cross-infection in the wards may reduce its incidence in the theatre, since some strains responsible for theatre infections originate in the wards (Gillespie *et al.*, 1959). The treatment of pre-operative bath water, and the provision of freshly disinfected bedding are perhaps of special value.

In the theatre itself, disinfectants have important uses in the treatment of the patient's skin before incision, and the surgeon's hands before he puts on his gloves. These applications are dealt with by Dr. Lowbury (see page 85).

FURUNCULOSIS IN NURSES

Recurrent furunculosis is usually the result of re-infection from a carrier site on the patient's body. Many nurses become carriers of 'hospital' staphylococci, and the furunculosis which in some hospitals causes a good deal of minor ill-health, is often a consequence of cross-infection.

Gould and Cruikshank (1957) prevented recurrences of furunculosis by treating nasal carriage with antiseptic creams. Tulloch, Alder, and Gillespie (1960) found that in a minority of patients the re-infecting staphylococci came from non-nasal sites such as the perineum (especially when boils affected the lower part of the body) and from superficial lesions which, although often apparently trivial, might be colonized by staphylococci, *e.g.*, external otitis, blepharitis, and eczematous areas. For maximum success in preventing recurrence they therefore recommend that treatment of nasal carriage with mixtures of neomycin and bacitracin (Neobacrin) or neomycin and chlorhexidine (Naseptin) in water-miscible creams should be supplemented by daily hexachlorophane baths and powder. Colonized areas of eczema, etc., were treated with creams or drops containing neomycin.

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The cleaning of ward floors and the bacteriological study of floor-cleaning machines

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SYNOPSIS Current trends in ward flooring materials and cleaning methods are considered from the point of view of the hospital bacteriologist. Methods employed in an investigation into the bacteriological safety of a number of floor-cleaning machines are described, and some considerations governing the choice of vacuum cleaners for ward use are discussed.

The ward floor has long been recognized as a secondary reservoir of infection (Cruickshank, 1935). The extent to which contamination from this source contributes to cross-infection is not clearly definable, though it is well known that the flora of the floor is often found to reflect that which besets the occupants of the beds (Garrod, 1944), and instances have been recorded in which it has been impossible to incriminate any other source of cross-infection (Rubbo, 1948). The avoidance of needless air contamination by the adoption of good housekeeping methods is basic practice in hospitals today, and if efforts to this end are to be rightly directed and economically achieved, the hospital bacteriologist must be prepared to advise those who have responsibility on the domestic and administrative sides. They in their turn have become increasingly conscious of floor-cleaning problems as the result of labour difficulties, work-study investigations, and pressures by those commercially interested in floors and floor cleaning.

Information on which to base such advice is not to be found amid the usual daily reading of most bacteriologists, and therefore the following summary of current developments in floor materials and cleaning methods may be found useful for reference. The bacteriologist is often asked to advise about the safety, from the cross-infection aspect, of vacuum cleaners and floor-cleaning machines, and lacking the time to develop a satisfactory technique for eliciting the necessary evidence, may fall back on methods which give results of which the accuracy is far removed from that found in his more ordinary investigations. An account is therefore given of methods used to study this problem in a recent investigation on behalf of the Division of Hospital Facilities of King Edward's Hospital Fund for London, the results of which are published elsewhere (Bate, 1961).

FLOORING MATERIALS

WOOD Of the types of flooring materials that have been used in the past for hospital wards, probably the most popular has been wood block or wood strip. Such flooring is undoubtedly attractive and long wearing, but it is porous, and the surface is broken between the strips or blocks by crevices which retain dust. The application of seals to these floors instead of polish is at present gaining popularity, though not all seals give equally satisfactory results. But there seems little doubt that these finishes will be generally used on account of their non-slip qualities, their effective filling of pores and crevices, and their labour-saving potentialities. There are about 40 different types of seal available at present, and the Timber Development Association has published an informative booklet about them.[1] This subject is also being investigated by the Division of Hospital Facilities of the King's Fund, and a report on the trials of various seals and polishes in hospital wards will in due course be made available.

MAINTENANCE OF SEALED FLOORS Sealed floors can be simply maintained by vacuum cleaners or impregnated mops, with damp mopping occasionally to clean any unusual soiling. The life of the seal may be prolonged by the application of a suitable emulsion polish, and this can be a factor of considerable importance in hospital wards, which may be in constant use for years on end without any opportunity for renewing the seal. Some seals are more difficult to apply than others, when the time comes for the renewal of worn areas. Thus the hospital's initial choice of seal should be determined not only by the

[1]The Timber Development Association's booklet on floor seals is known as Information Bulletin C/1B/3, and is published by the Association at 21 College Hill, E.C.4.

lasting qualities of the seal, but also by the standard of the staff available for re-applying it. It is not generally practicable to re-seal worn areas while the ward is occupied, partly because of the smell, and partly because of the need to shut off areas of the ward while the fresh seal is drying. The worn areas are always in the busiest parts.

LINOLEUM After wood, linoleum has probably been the most widely used flooring material for wards, either as continuous sheet or as tiles. It is semiporous, and is often wax polished, but can be effectively sealed to prevent the treading in of dirt and to make for easier maintenance. Non-slip, resin-emulsion polishes can be applied from time to time, as for sealed wooden floors; these need relatively little buffing, so that daily maintenance is confined to removing dust with vacuum cleaner or impregnated mop and removing adherent soiling by damp mopping with antiseptic detergent when required.

RUBBER Rubber flooring is still quite widely used in hospitals, and it has many good qualities. However, it is relatively expensive, and it seems possible that some of the newer types of plastic materials may provide as high a standard of flooring at lower cost.

PLASTIC FLOORING In more recent years, rapid progress has been made with the development of thermoplastic 'asphalt', vinyl asbestos, and polyvinylchloride (P.V.C.) flooring materials. Polyvinylchloride in particular seems to have considerable advantages. It has resilience and a naturally tough impervious surface, non-slip when dry. Adjacent sheets can be welded together to give a surface which is continuous from wall to wall. Emulsion polishes can be applied occasionally, otherwise daily maintenance needs only a vacuum cleaner or impregnated mop. It also lends itself particularly well to damp-cleaning and flooding techniques, which are discussed below. Vinyl asbestos has rather similar properties but cannot be welded. Thermoplastic 'asphalt' tiles are cheap and hard wearing, but perhaps less suitable for wards as they are relatively noisy and less resilient.

TERRAZZO This material is unlikely to be laid in wards to any extent, as it is hard, noisy, and unless underfloor heating is provided, cold. But if laid with regard to certain conditions (fall towards a gutter ensured, safeguards against leakage, use of built-in furniture cantilevered from the walls and clear of the floor) it can form the basis of a system of floor cleaning by flooding with water from strategically-placed nozzles. Such a system has been in use for six years in the Out-patient Department of The Hospital for Sick Children, Great Ormond Street, London. In that setting it has worked well, and it is conceivable that the system might find an application in special places, such as dressing-rooms, where the need to apply stringent cleaning methods at frequent intervals was felt to outweigh the obvious difficulties.

CURRENT TRENDS IN FLOOR CLEANING METHODS

VACUUM CLEANING AND POLISHING The danger of sweeping with ordinary brooms needs no mention here, but sweeping mops and cloths impregnated with dust-retaining oils have recently been introduced as alternatives to the almost universal vacuum cleaner. It is said that these are efficient sweepers that do not disturb floor dust, but whether they remove it as efficiently as vacuum cleaners do, and whether they tend to leave behind a film of oil which may in time make the floor retain dust are questions which are not yet answered. Vacuum cleaners, on the other hand, leave no doubt of their efficiency when they are properly maintained. The same cannot be said of suction polishers in their present form. One type of these machines has rotating brushes which tend to cause centrifugal dispersal of dust unless the surrounding flanges are adjusted accurately to the floor. Most domestics have no idea that these flanges are anything other than buffers. The cloth dust bags are quite ineffective in retaining bacteria-carrying particles (Bate and James, 1958), and though paper bags are now fitted as well, these are not often emptied, and are frequently found to be torn and useless.

So far as hospital wards are concerned, it is most desirable that the use of floor-polishing machines should be reduced to a minimum, partly because of the noise and disturbance they cause, and partly because of the time it takes to carry out the polishing procedures with these machines. In this connexion, the production of self-burnishing emulsion polishes is a welcome development that may lead to greater simplification of maintenance routines for linoleum, rubber, and plastic floors and for sealed wood floors. These polishes are applied to the floor by spray, lambswool applicator, or similar device, and are said to need no mechanical burnishing at all, and renewal only at intervals of several weeks, depending on traffic.

WET CLEANING AND DRY CLEANING Damp mopping can be highly effective so long as the mops are kept clean and enough antiseptic detergent is used to ensure effectiveness up to the end of the operation. An improvement on this technique is that of flooding and wet pickup, originating in the United States.

This can either be part of a regular routine as a supplement to damp mopping, or reserved for terminal disinfection. After removing the furniture, several litres of an antiseptic detergent solution are poured on to the centre of the floor and spread out to the walls by a clean mop, and subsequently dried off by a wet pick-up vacuum cleaner. Machines of this kind, having a hose and nozzle, are now available in Great Britain, and seem more convenient for the purpose than are the ordinary floor-drying machines.

Wet cleaning is damaging to a wax-finished floor, and is not considered advisable on sealed floors except for the occasional removal of heavy soiling. Flooding is obviously impracticable on porous or discontinuous surfaces such as timber and linoleum, where the solution may seep through. It should be reserved for impervious surfaces such as terrazzo, welded P.V.C., or other jointless composition, though it has been successfully used with plastic tiles where these have been expertly laid with waterproof adhesive.

Where wet cleaning or flooding may damage the floor surface, it may be practicable to use a dry-cleaning technique that involves the sprinkling or spraying of an emulsion polish on to the floor, followed by the operation of an electric polisher with a nylon or steel wool pad. This method has appeared to be successful in some hospitals, and is claimed to combine cleaning and polishing in one operation; but its bacteriological effectiveness seems to need further investigation.

SOLUTIONS SUITABLE FOR DAMP CLEANING AND FLOODING The 'black fluid' phenolic antiseptics and liquor chloroxylenolis both contain soaps and therefore have detergent as well as variable antiseptic properties, but neither is very suitable for this purpose, the one on account of its smell, and the other of its expense, and there is a need for a cheap, effective and persistent detergent antiseptic for floor hygiene. Two developments from the United States seem promising. Adams, Fahlman, Dube, Dube, and Read (1959) found a demonstrable antibacterial effect for as long as eight hours after vigorous cleaning of surfaces with a mixture of sodium benzene sulphonate, *o*-phenylphenol, and *p*-tertiary amylphenol. The iodophors, which are mixtures of iodine with non-ionic surface-active agents, as well as being detergent, are said to have powerful antiseptic effects, and to remain lethal to *Staph. aureus* and *Salm. typhi* in five minutes down to a dilution of 6 parts of iodine per million (Gershenfeld and Witlin, 1955, quoted by Sykes, 1958). A solution containing 75 parts of iodine per million has been successfully used in the flooding technique described above.

THE SAFETY OF FLOOR-CLEANING MACHINES

As has already been pointed out, among much that is changing in floor-cleaning practice, the use of vacuum cleaners for removing dust remains almost universal. Such machines have potentialities for spreading infection, and it is important to be sure that machines in use or under consideration for purchase are above suspicion in this respect. The bacteriologist asked to report on the bacterial content of the exhaust air may find it all too easy to hold a culture plate in the air stream for a short time, and later to give a report on the significance of the resulting growth. Such a proceeding is unwise for a number of reasons beside the absence of any standard for comparison. Accurate sampling is governed by very precise conditions, none of which can be observed by this makeshift method. Furthermore, the emerging jet is of high velocity and low pressure in relation to the surrounding air, which rushes in from all sides to mix with it, so that the sample must include a quantity of air which has not passed through the cleaner at all. This phenomenon, an example of the Bernouilli effect, is illustrated in Figure 1.

There is, in fact, no accurate method for sampling the bacterial content of air which is moving at anything up to 80 miles an hour. The sampling of even slow-moving air brings in aerodynamic problems which most bacteriologists will prefer to avoid. Recognition of this fact led to the conclusion that the simplest means of sampling the output from a number of vacuum cleaners would be to trap the air in a reservoir and hold it stationary while a sample was collected by a slit sampler (Bourdillon, Lidwell, Lovelock, and others, 1948).

Figure 2 illustrates the apparatus, which consists of a reservoir of approximately 20 cu. ft. capacity, made out of a 4 ft. length of heavy gauge polyethylene 'layflat' tube 9 ft. in circumference. The upper end is closed by pleating and firmly tied with string, which also suspends the bag from the ceiling. The other end is open and fixed to a stout wooden frame which can be fastened down on to the surface of a table which forms the base of the reservoir (Fig. 3).

The slit sampler stands on this table, and is thus inside the reservoir, the electrical leads and suction line being carried down through airtight openings in the table top to the air pump on the shelf below. In use, the reservoir frame is held down on to the table top by light plastic clips, strong enough to maintain an airtight joint between the surfaces, which are covered with strips of plastic foam draught excluder, but able to release the bag if overdistended. Air from vacuum cleaners under test fills the reservoir through a lightweight polyethylene tube 4 ft. in diameter and

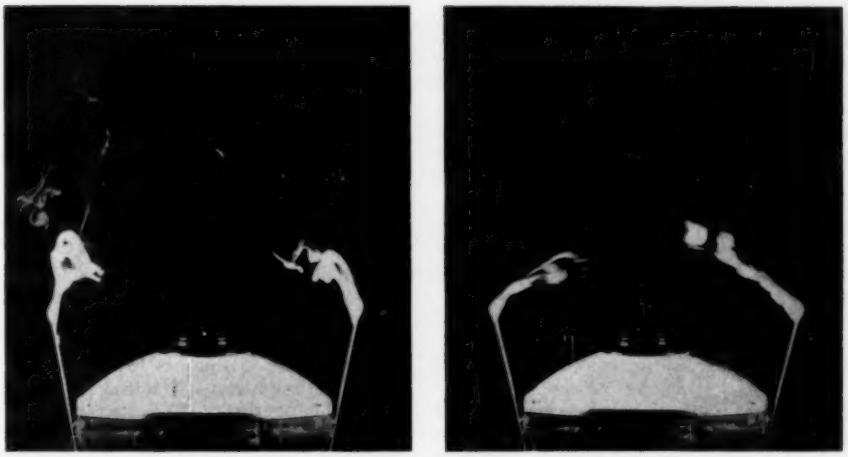

FIG. 1. *Aspiration of surrounding air into the exhaust jet of a cylinder-type cleaner. On the left, the motor is stationary.*

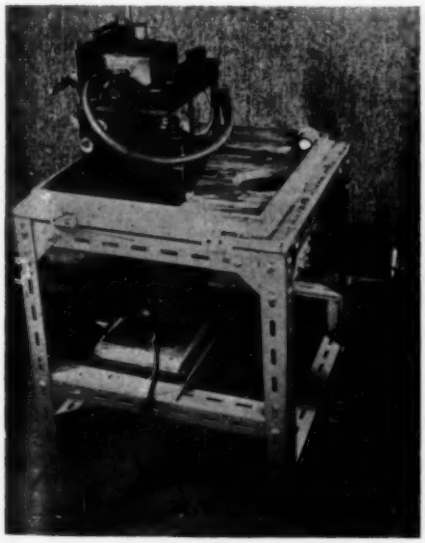

FIG. 3

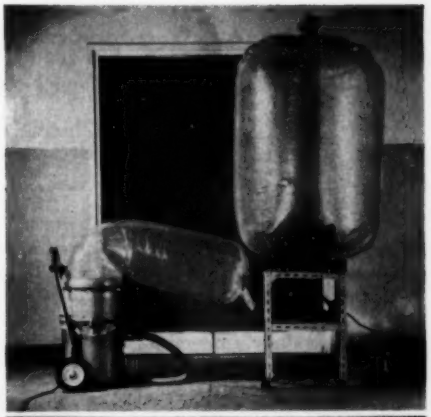

FIG. 2

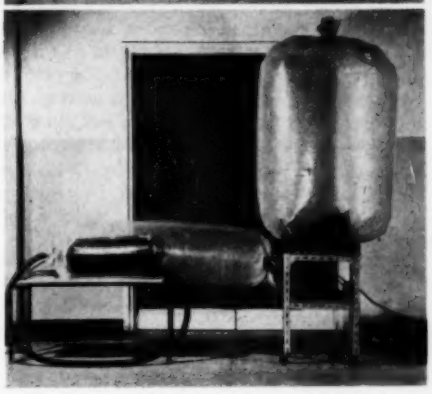

FIG. 4

FIG. 2. *The apparatus in use, collecting exhaust air from a large tank-type vacuum cleaner.*

FIG. 3. *The table which carries the slit sampler and forms the base of the air reservoir. To the right of the slit sampler is the opening of the collecting duct.*

FIG. 4. *Collection of air from a small cylinder-type vacuum cleaner.*

5 ft. long attached to the metal duct leading to the interior of the reservoir through the opening in the table top beside the slit sampler. Into the free end of this bag can be tied by means of a length of rubber tubing, as seen in Fig. 2, the outlet of the largest machine examined, the whole of a cylinder-type machine (see Fig. 4) or of a suction-polisher, of which the brushes alone remain outside. The sequence of operations in a test run is as follows:

1 To ensure a free flow of air through the machine under test, compacted masses of dust and fluff are removed from the dust container, but dirt clinging to the filtering surfaces is not disturbed.

2 The cleaner is connected to the electricity supply and fastened inside the receiving tube.

3 The slit sampler is loaded with a plate of culture medium.

4 The reservoir is clamped down and evacuated of all contained air by means of a second vacuum cleaner whose nozzle is inserted through a second opening in the table top, normally kept stoppered.

5 The cleaner under test is allowed to run for two minutes, driving out any residual air and any dust particles from the inside of the apparatus.

6 The joint between the reservoir and the table top breaks to allow the escape of surplus air, but as the cleaner is switched off, the reservoir is clamped down to hold the last 20 cu. ft.

7 A 5 cu. ft. sample is then run through the slit sampler, the switch for this being outside the reservoir.

In order to discover whether the apparatus was capable of demonstrating the relative filtration efficiency of a number of cleaners, some preliminary runs were carried out on machines which could be expected to yield air in descending grades of purity relative to one another. An old cylinder cleaner with one layer of filtering material gave four colonies in 5 cu. ft. An older domestic vacuum cleaner of the hanging-bag type gave 66 colonies in the same volume of air, rather more than the number obtained from a sample of the air in the room. Lastly, a cylinder-type cleaner was stripped of all its filtering media, and in place of the dust bag there was fitted a cloth bag made of 2 mm. mesh curtain netting. This bag was filled with the dust from the bag it replaced. A 5 cu. ft. sample of the air yielded 510 colonies. The fouling of the apparatus resulting from these and subsequent tests was removed by washing the polyethylene tubes with soap and water and polishing with an antistatic polish.

In the actual investigation, the sequence of operations described above was performed three times for each cleaner at each test. No dirt was drawn in, tools were detached, and the air entry kept well above floor level.

AIR DISTURBANCE The high-velocity jet discharge of many vacuum cleaners has already been mentioned, and a method was also devised to assess and compare the extent of air disturbance caused by each. In a small room, 10 ft. by 12 ft., furnished only with a bed, locker, chair, and chest of drawers, the slit sampler without the reservoir was set up on one side of the bed. The cleaner under test stood on the other side, between the bed and the window. To eliminate disturbance due to suction, the end of the hose was hung out of the window, the resulting gap being filled with broad adhesive tape. The operator sat at arm's length from the sampler, moving only as much as was necessary to change plates, having switches at hand to control both the cleaner and the sampler. After allowing 15 minutes for disturbance due to setting up the apparatus to subside, a 5 cu. ft. sample of room air was taken. The cleaner was then switched on for four minutes, switched off and a second sample of room air taken. Six further samples were taken at intervals of 15 minutes during the period of one and a half hours from the operation of the cleaner. Colony counts demonstrated the degree of air disturbance, and its subsequent settling.

CHOICE OF A VACUUM CLEANER The results obtained by these methods have been fully reported elsewhere (Bate, 1961) and need not be repeated here, but in the light of this work it is possible to make some suggestions as to the choice of vacuum cleaner for ward use. The first consideration should be the method of finally disposing of the collected dust, and preference should be given to those machines in which a disposable paper bag is provided for this purpose. Secondly, performance figures should be studied; they are readily available. A high rate of air displacement is desirable; a high closed vacuum is less important, but the most satisfactory index of performance is the figure for suction power, measured in foot-pounds per second according to a formula laid down by the British Standards Institution (1958). This is now available for most cleaners. Filtering efficiency may be considered adequate if there are not less than two layers of filtering material actually within the body of the machine, on the input side of the air impeller, and separate from one another so that failure of the first layer is immediately apparent on inspection of the second. Finally, the exhaust jet should be well diffused, and any diffusing device should preferably form an integral part of the machine, or be detachable only with difficulty.

I am very much indebted to Mr. M. C. Hardie, of the Division of Hospital Facilities of King Edward's Hospital Fund for London, for putting at my disposal a very large amount of information about floors and their cleaning.

The slit sampler used in the investigation was lent to me by the Wright-Fleming Institute of Microbiology, St. Mary's Hospital, to which I am most grateful; as also to Professor R. E. O. Williams for his helpful criticisms.

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Sterilization by dry heat[1]

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SYNOPSIS The advantages and disadvantages of three forms of dry heat sterilization are discussed. In addition a fourth method, consisting of heating by infrared rays *in vacuo*, is described. This method is particularly suitable for instruments used in the operating theatre, since it can replace an autoclave where a supply of steam is not available. Recommended times and temperatures for dry heat sterilization are detailed, and are related to the thermal death point of *Cl. tetani*. The dangers of recontamination during the cooling process are discussed.

Sterilization by dry heat has become increasingly popular in Great Britain. This is probably for two reasons: first, the need to sterilize small objects, particularly syringes, and secondly, the apparatus required is comparatively inexpensive when compared with the autoclave. It should also be added that shortly after the last war autoclaves were in short supply, and a number of people became increasingly interested in dry heat sterilization. Before considering the apparatus available it is as well to discuss the advantages and disadvantages of the method. It must be emphasized that sterilization by dry heat depends upon the penetration of adequate heat to the article as a whole; thus it is possible to sterilize such objects as syringes already assembled and presealed in their container, whereas steam and gases, such as ethylene oxide, can only be relied upon to kill organisms if the steam or gas comes into direct contact with the surface of the objects. Steam, therefore, is not suitable for such equipment as assembled syringes or for articles enclosed in a sealed tube. Another advantage of this method is that objects which are damaged by water or steam, e.g., powder or ointment, can be sterilized provided the heat penetrates to all parts of the substances. The disadvantages of the method are that, first, some of the apparatus, such as hot air ovens,takes a considerable time to reach sterilizing temperature, and the temperature and time necessary for sterilization must be considerably higher and longer than for steam. Secondly, much of the apparatus in use is unreliable and shows considerable variation in temperature during sterilization. Thirdly, the objects, particularly metal, may become oxidized at high temperatures or they may not withstand the temperature.

[1]From a lecture given by one of us (E.M.D.) to the School of Pharmacy, Chelsea College of Science and Technology, London, on 2 February 1960.

APPARATUS

The apparatus necessary to provide dry heat for sterilization can be considered under four headings. First, the hot air oven; second, the conveyor oven; third, conducted heat; and fourth, dry heat in the presence of a vacuum. It is proposed to examine each of these methods more critically.

1 HOT AIR OVEN Hitherto the most popular method for dry-heat sterilization has been the hot air oven. However the limitations of this apparatus have already been fully discussed (Ewald and Schmid, 1953; Darmady and Brock, 1954). These investigations showed quite clearly that a number of hot air ovens examined could not be relied upon to provide an even temperature throughout the oven, and indeed might not reach the temperature recorded by the thermometer. Thermocouples placed in syringes showed that there might well be a variation in temperature of 30 or 40°C. (Fig. 1). Investigations also showed that the time taken to reach the sterilizing temperature could be reduced if the oven was allowed to heat up first, and, if this was done, the temperature variation in the articles to be sterilized was not so great. Furthermore, it was shown that the provision of a fan shortened the time taken to reach sterilizing temperature and that the forced circulation of air ensured that the variation was reduced to a minimum (Fig. 2). Nevertheless, in spite of the improvement obtained by having a properly designed oven with a fan, the methods of loading were important, for it was found that unless objects were loosely packed, there would be a delay in heat penetrating to the centre of the load. It is essential, therefore, that every oven should be tested with thermocouples to determine the time taken for larger objects, such as petri dishes in copper canisters or containers holding vaseline, to reach the sterilizing temperature. Recently the British Standards Institution has laid down criteria for hot air sterilizing ovens. The specifications include the need to test the oven with a predetermined load, and that the temperature variation shall not exceed 5°C. at any time within 60 minutes after the heating-up period. Such ovens

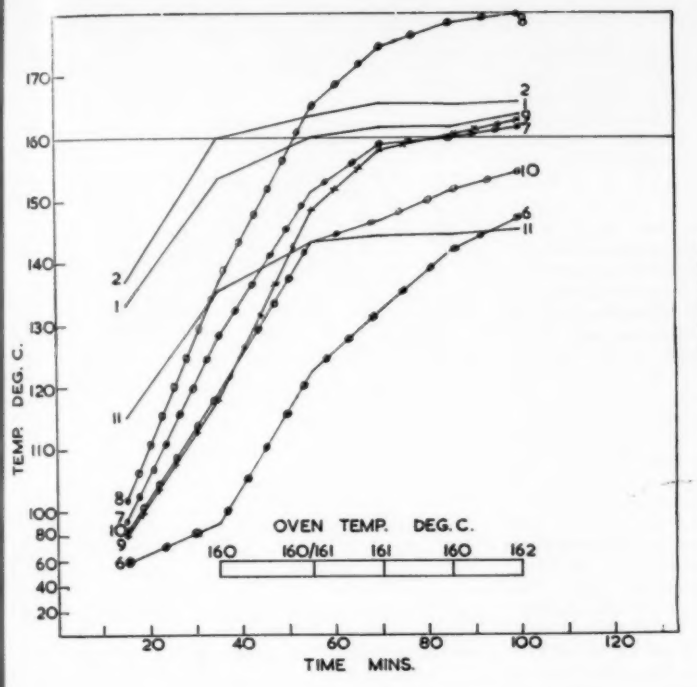

FIG. 1. *Temperature recordings in syringes sealed in aluminium containers in a gas-heated oven without a fan heated to 160°C. and loaded hot. Note that the variation of temperature was 40°C.*

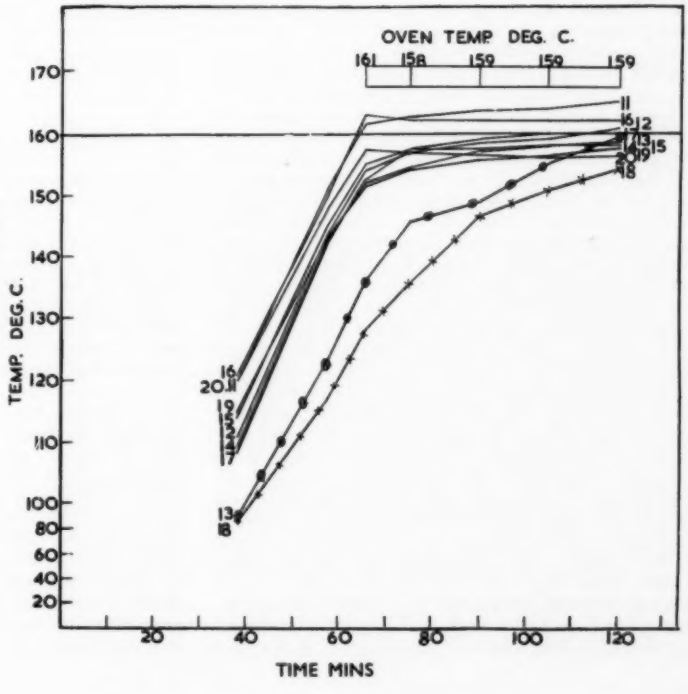

FIG. 2. *Thermocouple recordings in syringes sealed as before in an electrically-heated oven fitted with a fan. Curves 13 and 18 denote tightly-packed syringes. With the exception of these syringes the overall variation was not more than 5°C. from 160°C.*

are particularly necessary for the sterilization of instruments or syringes where it is imperative that the overall variation of temperature should be reduced to a minimum. The principal disadvantage of the hot air oven is the time taken by the load to reach the sterilizing temperature and the danger that articles can be removed from the oven before the process is complete.

2 CONVEYOR OVEN The design and performance of the conveyor oven has been described earlier (Darmady, Hughes, and Tuke, 1957; Russell and Abdurahman, 1960). This apparatus consists of an insulated tunnel in which a series of 'infrared' heaters has been placed at predetermined positions. A metal moving belt carries articles to be sterilized. The advantages of the apparatus are, first, that it enables a continuous flow of work to be produced, and is therefore applicable to a syringe service which is processing at least 600 syringes a day. Secondly, articles of standard size receive the same heat treatment for the same length of time. Thirdly, because of the 'infrared' type of heaters used, heating up is reduced to the shortest possible time. Fourthly, the machine is simple in design and there is virtually nothing to go wrong, since the operator merely places the objects on the moving belt, and once it enters the tunnel .nere is no possibility of interference. It is, however, important to remember that the machine has been designed to sterilize a fixed standard load, and articles submitted to it will receive an equal amount of heat. Both the mass of the syringe and the surface of the outer container have a direct bearing on the amount of heat absorbed by the article to be sterilized. Thus when a 2 ml. syringe was placed in a glass test-tube the syringe reached a temperature of 180°C. in less than two minutes, whereas if the same size of syringe were placed in a polished aluminium container the time taken would be eight minutes. Again, if a large 20 ml. syringe were submitted to the same heat source and placed in a similar container the centre of the syringe would take a longer time to attain the required temperature than those of the smaller size. It is, therefore, possible that by treating the outer surface of the container in an appropriate way all the syringes will receive the same treatment for the same length of time. To ensure this in the Portsmouth Syringe Service, 2 and 5 ml. syringes are sterilized in a grey anodized aluminium container and the 10 and 20 ml. syringes are sterilized in a black anodized container. It is apparent that by altering the surface and colour of the container many other articles could be sterilized, provided the overall load is not exceeded. For example, four dressing forceps placed in a grey anodized 20 ml. container will reach sterilizing time at the same moment as a 20 ml. syringe in a black anodized container. Care should, however, be taken to ensure that the temperature achieved does not exceed 210°C. as the instruments may become oxidized during the cooling process. Examples of temperature recordings are shown in Fig. 3.

With these machines it is important that the voltage remain constant and they should all be fitted with a voltage stabilizer, since it has been shown that the temperature will vary by the square of the voltage variation. Similarly, the heat uptake in the syringes or instruments

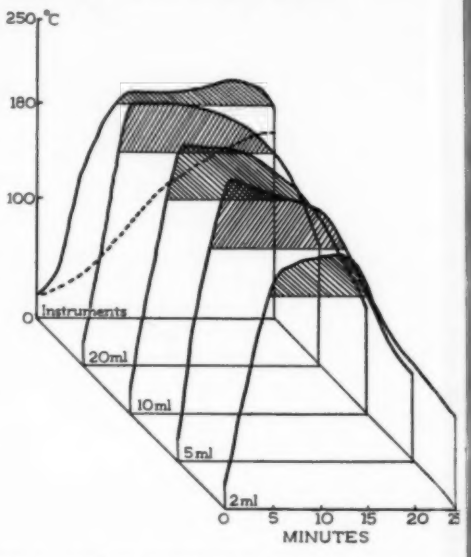

FIG. 3. *A three-dimensional graph showing the heat taken up of four syringes of different sizes and instruments in their containers during their passage through a conveyor oven. The hatched areas denote the time and level of temperature exceeding 180°C.; 2 and 5 ml. syringes were placed in dulled aluminium containers and the 10 and 20 ml. syringes in black anodized containers. The dotted line denotes instruments placed in polished aluminium containers, and should be compared with those in grey anodized containers.*

will vary by the square of the distance from the heat source. The advantage of these machines is that they are mechanically sound and there are few parts to go wrong. In practice the elements, which are continuous strips, last for long periods of time, and indeed the machine used in the Portsmouth Syringe Service has only required new elements once during its life of six years. The only other mechanical failure that is likely to occur is in the electric motor used for the moving belt. The principal disadvantage of this machine is that the load cannot be varied and it is not suitable for the sterilization of instruments for the theatre, since the numbers and types will differ.

(3) CONDUCTED HEAT. In a previous communication, Darmady, Hughes, Jones, and Tuke (1958) described experiments with an apparatus suitable for conducted heat sterilization. This consisted of a thermostatically controlled hot plate on to which an aluminium block had been fixed. Holes were bored to take six 2 ml., two 5 ml., and one 10 ml. Nuffield containers. Obviously these holes can be varied to suit individual needs. Cutting instruments were placed on a flat tray fitted with a lid on the top of the block. It was covered by a hinged, resin-bonded, fibreglass insulating cover. Investigations showed that the temperature variation when tested by thermocouples was

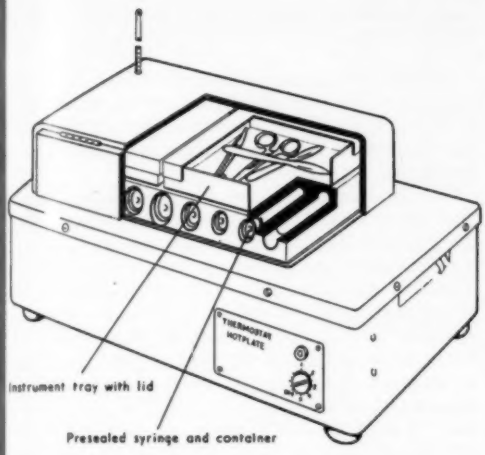

FIG. 4. *A thermostatically controlled hot plate with an aluminium block used for the sterilization of small numbers of instruments and syringes in their containers.*

±1°C. and so provided the most accurate form of controlled heat that has yet been found (Fig. 4). In the apparatus described, the trays were placed on top of the hot plate, and it was shown that instruments laid flat in them could easily be sterilized. This form of apparatus is particularly suitable for those who wish to provide heat at a precise temperature or to sterilize a small number of instruments or syringes. To save time it was found that it was better to set the aluminium hot plate at a standard temperature (190°C.) and maintain it continuously. In the machine described it was found that a syringe in its container could be sterilized in 22 minutes. Since then, further experiments have shown that the container need not fit the bore holes accurately, and will be adequately sterilized in the same time.

HIGH-VACUUM, INFRARED STERILIZER

The three types of apparatus previously described have not proved suitable for sterilizing operating instruments, first because the hot air oven takes time to reach the required temperature and the working space is usually restricted; secondly, because the conveyor oven is not suitable for varying loads and may cause oxidation of the instruments, and thirdly because the capacity of conducted heat equipment is strictly limited. Although steam at high pressure is suitable for the sterilization of instruments, a survey of the hospitals in the Wessex Regional Hospital Board area showed that about one-third were not equipped with a steam supply, and that designers of modern hospitals, particularly in tropical countries, were anxious, since central heating was not required, not to include one. One architect stated that to equip a hospital with a special steam supply to the theatres might cost as much as £75,000.

It was therefore decided to try and devise equipment which would be capable of sterilizing instruments by dry heat using electrical power. Such a piece of apparatus has now been developed.

It consists of a double-ended rectangular chamber measuring 30 in. × 14 in. × 5½ in. (clear) fitted with a tray capable of carrying on average 70 instruments and two stainless steel bowls 14 in. in diameter. The roof and floor of the sterilizer are fitted with four infrared heaters of 1 kW. and four of 0.5 kW. respectively (eight heaters in all). The cycle, which is completely automatic, is as follows:—

1. After loading the chamber the doors are closed, and the starter switch engaged. To prevent accidental contact the switch has to be held for at least 10 seconds.
2. A vacuum pump now exhausts the chamber to a pressure of 1 or 2 mm. Hg absolute pressure. As soon as this is reached, and if it is obtained (usually in three and a half minutes), the heating elements are switched on, the vacuum maintained, and the heat applied. The temperature of the chamber was found to reach a maximum of 280°C.
3. At the end of the predetermined time of heat treatment, the heaters are turned off and the vacuum broken by refilling with nitrogen gas through a filter from a cylinder. This fulfils a twofold function: first it ensures that the instruments are not oxidized during the cooling cycle and secondly it helps to cool the instruments so that they can be handled with safety. In one model, a further vacuum was drawn and again replaced by nitrogen. This, however, did not greatly improve the cooling.

In order to determine the efficiency of the machine and to provide a satisfactory time cycle, a number of investigations were carried out. The method of testing the apparatus was as follows:—

EXPERIMENT 1 The tray was fully loaded with at least 70 stainless steel instruments or syringes. A number of these were smeared with either dried soil suspended in serum and allowed to dry at room temperature or with *Cl. tetani* spores suspended in broth dried in a similar manner. Many of the instruments were so arranged that infected instruments were shielded from direct radiation from the heaters. In the first machine heaters were provided only on the roof of the chamber, and although the cycle was exactly as described, the heat treatment was applied only for two to three minutes.

In six consecutive experiments carried out over a number of days on 61 instruments and nine syringes infected with soil, only 10 and eight respectively proved to be sterile; of 12 syringes and slides infected with *Cl. tetani*, N.C.T.C. 5411, all proved to be sterile.

EXPERIMENT 2 The machine was modified so that four heaters were placed on the roof and four on the floor of the chamber. Heat treatment was applied for two to three

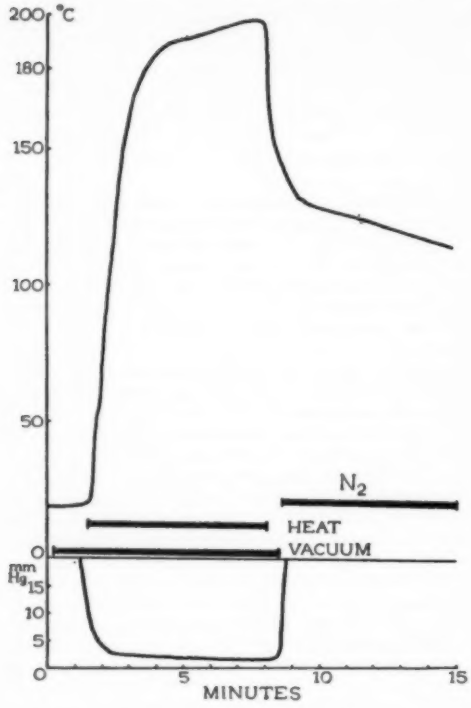

FIG. 5. *The temperature recordings of a typical cycle of the high-vacuum, infrared sterilizer.*

minutes as in the previous experiments, and the instruments were loaded as before. Out of the 18 infected instruments with spore-bearing soil only 11 proved to be sterile.

EXPERIMENT 3 For this investigation the same machine as described in experiment 2 was used, but the heat treatment was increased to seven minutes. All the 100 instruments infected with *Cl. tetani* proved to be sterile, although one out of 12 infected with soil gave a positive culture.

EXPERIMENTS 4 AND 5 The same machine was used, but the heat treatment was reduced to four and six minutes respectively. With the cycle set at four minutes, 144 out of 153 tests using *Cl. tetani* and at six minutes, 35 out of 37 using dried soil, proved to be sterile. For this reason it was decided that it was necessary to provide the extra safety margin and therefore the time cycle should include heat treatment for at least seven minutes, making a total cycle of 15 minutes. Temperature readings are shown of a typical run in Fig. 5. Unfortunately it was not possible to obtain temperature recordings of the instruments *in situ*. However, it was clear from our investigations that using the time cycle recommended the surface heat was more than adequate to ensure complete sterility.

The repeated heat treatment has so far not caused properly made stainless steel instruments conforming to B.S.I. standards to deteriorate, although some of the instruments have been exposed on 100 or more occasions.

The makers claim that the advantages of this machine are that the provision of a vacuum of 2 mm. Hg absolute during the heating cycle prevents first, the convection of currents normally found in an hot air oven and responsible for heat variation in the chamber; second, a build-up of pressure within the chamber so that the chamber and doors need not be built to withstand it; third, oxidation of the instruments. Furthermore, the heating process is accelerated, since heat is not taken up by the air. Throughout the experiments care was taken to provide varying loads of instruments, and the infected instruments were placed in such positions that they were shielded from direct radiation. It is apparent that many of the smaller articles will reach the required temperature more quickly than larger articles, but as the temperature of the heaters is constant, the danger of overheating is minimal. From the experience gained it seems that this apparatus would be suitable as a sterilizer when high pressure steam is not available, and has the advantage that the time of the sterilizing cycle is not greatly increased over that of the high-steam sterilizers on the market at present.

BACTERIOLOGICAL CRITERIA

At the time of the introduction of the conveyor oven, the normally accepted standard for sterilization by dry heat was a holding time of one hour at 160°C. However, a survey of the literature (Darmady and Brock, 1954) showed that the recommendations varied considerably (Table I), and it was decided to investigate the problem, since it was clearly not practicable to provide a conveyor belt to maintain a sterilizing holding time for one hour at 160°C. It was therefore considered vital to sterilize the instruments at a higher temperature for shorter periods of time. For the purpose of this investigation, the thermostatically controlled hot plate fitted with an aluminium block was used, as has been described earlier and in detail elsewhere by Darmady, Hughes, Jones, and Tuke (1958).

Before each test, the thermostat was set at the desired temperature, and checked with thermocouples. The test organisms were dried on glass slides and placed in small Pyrex test-tubes plugged with cottonwool. The tubes were placed in the holes of the

TABLE I

TEMPERATURE LEVELS FOR HOT-AIR OVENS

Name	*Hot-air Sterilizer*
Bigger, J. W. (1949)	160°C. for 1 hour 140°C. for 3 hours
British Pharmaceutical Codex (1949)	150°C. for 1 hour
British Veterinary Codex (1953)	150°C. for 1 hour
Burrows, W. (1949)	170°C. for 2 hours
American Public Health Association (1950)	160°C.–180°C. for 2 hours
Dubos, R. J. (1948)	160°C. for 90 minutes
Fairbrother, R. W. (1953)	160°C. for ½–1 hour
Gerhards, G. A. (1952)	140°C. for 1 hour (electric fan oven)
Gradwohl, R. B. H. (1948)	170°C. for 2 hours (gas oven)
M.R.C. Memorandum No. 15 (1945)	160°C. for 1 hour from cold
Mackie, T. J., and McCartney, J. E. (1953)	160°C. for 1 hour 180°C. for ½ hour
McCulloch, E. C. (1945)	160°C. for 1 hour
Smith, D. T., and Martin, D. S. (1948)	150°C.–160°C. for 1 hour
Stitt, E. R., Clough, P. W., and Branham, S. E. (1948)	160°C. for 90 minutes (syringes must be separated)
Walter, C. W. (1948)	160°C. for 1 hour if instruments are clean 160°C. for 4 hours if oily or greasy
Whitby, L. E., and Hynes, M. (1951)	160°C. for 1 hour
Wilson, G. S., and Miles, A. A. (1946)	140°C.–160°C. for 1–1½ hours 400°C. for 20–30 seconds
Weedon, B. (1941)	320°F. for 1 hour (electric oven without fan) (160°C.)

aluminium block. The time taken to reach the desired sterilizing temperature was found on each occasion to be nine minutes, the test tube then received heat treatment for varying lengths of time.

In all 1,472 tests were made and three groups of organisms used, the results being compared with those obtained from the literature (Darmady, Hughes and Jones, 1958). Although dried soil was found to be most resistant, it was thought more reasonable to relate any recommendation for time and temperature to the behaviour of *Cl. tetani*, which, in our hands, proved to be the most resistant of all pathogens to dry heat. The results showed that although two strains of non-toxigenic *Cl. tetani* (N.C.T.C. 5411 and 5413) were used, there was no difference between them. Four hundred and seventy-four tests showed that sterility was attained by exposure to 180°C. for one minute, to 170°C. for five minutes, to 160°C. for 12 minutes, and to 150°C. for 30 minutes. For this reason it was decided to base a safety margin on the results with *Cl. tetani* and that this margin should be one and a half times that which is needed to kill the organisms.

However, as the heat of objects placed in a hot air oven may vary as much as 5°C. it was thought that an added safety margin was imperative. Thus it was recommended that the holding time for the selected sterilizing temperature should be one and a half times that required to kill all organisms at a temperature 10°C. lower than the temperature selected. For example, if the instruments were to be sterilized at 170°C. the holding time, exclusive of heating-up time, should be 18 minutes, or one and a half times the minimum lethal time (12 minutes) for *Cl. tetani* at 160°C.

Although the ability to kill a selected organism may be regarded as a satisfactory method of testing the efficiency of dry-heat sterilizing, there is a need to provide a visual and easy check for those employed in a central sterilizing service. For this purpose the Browne tubes III and IV have proved to be satisfactory, and a comparison is shown in Table II which sets out the recommended sterilizing times and the time taken for the Browne tubes III and IV to change colour. For the conveyor oven Browne's tubes No. IV are recommended as they more nearly conform to the recommended times and temperatures as set out in Table II.

TABLE II

Temperature	*Time Recommended (min.)*	*Colour Change in Minutes*	
		Browne Tube III	*Browne Tube IV*
160°C.	45	51[1]–61	42–51
170°C.	18	26–31	21–33
180°C.	7½	17–22	13–19
190°C.	1½	7–11	4–10

[1] The first figure in each column denotes the time taken for the first tube listed and the second figure the last tube of 10 to change colour

RECONTAMINATION

Recently, Russell and Abdurahman (1960) have drawn attention to the possible risk of recontamination of syringes during the cooling process immediately after sterilization in the conveyor oven.

They have shown, for example, that a 20 ml. syringe container can aspirate 70 ml. of unsterile air while cooling, thus drawing attention to the absolute necessity for adequate sealing of the containers. It is equally clear that this kind of recontamination might occur with other kinds of dry-heat sterilized equipment. In order to test the possibility of this recontamination, the following experiment was undertaken:—

Four sizes of containers were loaded with syringes and a piece of filter paper placed on top of the plungers. They were sealed with aluminium foil capsules and sterilized in the conveyor oven in the usual way. Other containers were processed in a similar way, but before being put into the oven the capsules were perforated by a needle prick. As soon as they emerged from the oven they were all placed in a container containing an aerosol of *Serratia marcescens* and allowed to cool. The seals were then removed and cultures made of the filter papers. Positive

cultures were obtained only from those syringes in which the cap had been deliberately punctured. In a further experiment, an inexperienced operator was asked to seal the containers, and they were sterilized and allowed to cool in a similar manner and once again positive cultures were obtained. However, those sealed in an automatic machine giving a fixed pressure or by an experienced operator ensured that recontamination did not occur. Russell and Abdurahman (1960) have suggested that although the possibility of recontamination is slight this can be overcome by placing a piece of cottonwool over the top of the syringe and sealing into position. This, however, is not desirable as small fibres may become jammed between the plunger and barrel causing the well-known horse shoe fracture of the barrel. This is particularly liable to occur in syringes that have been lubricated. Alternatively, these authors have suggested that cooling under high-intensity ultraviolet light would prevent recontamination.

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Prevention of infection in operating theatres

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Sepsis arising at the time of operation has proved easier to understand than some other forms of cross-infection. Conditions in the operating theatre are normally straightforward: usually only one patient is exposed, and the surgical team consists of a restricted number of doctors and nurses, who by tradition follow a strict routine. In this paper we have summarized, in a somewhat dogmatic form, our present views on operating theatre design and practice insofar as they affect the prevention of infection. Staphylococcal infection, clostridial infection, and infection of the urinary tract with miscellaneous bacteria will be our chief topics, with particular emphasis on those features that have been clearly shown to be effective in prevention. It is, of course, likely that with further experience the emphasis will change and some of the preventive measures that, for lack of precise facts, we have barely mentioned may later assume greater prominence.

STAPHYLOCOCCAL WOUND SEPSIS

RECORDS The only reliable method by which the surgeon can find out how many of his wounds heal without sepsis is to arrange for the keeping of records specifically for the purpose; impressions are often quite misleading. How and where the records are kept is less important than acceptance of the principle that they are worth keeping. An attempt should be made to distinguish infection arising at the time of operation from infection originating subsequently. In general, pus present at the first dressing, pus deep in the wound, and fever early after the operation suggest infection in the operating theatre, although the distinction may not be an easy one to make and may not be possible at all. Records based solely on laboratory results are liable to be misleading because the indications for sending specimens to the laboratory will vary with circumstances, and because clinical signs of disease are more important than positive bacteriological cultures. The responsibility for keeping the sepsis record should be carried by the surgeon in charge of the patient, or by a member of his team.

SEPSIS ARISING FROM SURGEONS WITH SEPTIC LESIONS While it is true that there are innumerable instances of surgeons operating while suffering from furunculosis or other septic lesions with no apparent harm, it is also true that this practice has led to catastrophe. McDonald and Timbury (1957) and Mitchell, Timbury, Pettigrew, and Hutchinson (1959), for instance, both reported outbreaks of sepsis from this cause and unless there is some absolutely compelling reason surgeons with active sepsis should not operate.

SEPSIS ARISING FROM CARRIERS The evidence that a healthy staphylococcal carrier in the surgical team can infect a wound at the time of operation is of two sorts. In a pioneer paper Devenish and Miles (1939) described an outbreak of post-operative wound sepsis in which nearly all the infections occurred in patients operated on by one of the four surgeons in the team. The surgeon concerned was a healthy nose and hand carrier of *Staph. aureus*, and Devenish and Miles thought that the most probable route of infection was through the permeable sleeves of his gown or through glove punctures. Phage typing has confirmed circumstantial incrimination of carriers in several more recent studies. Blowers, Mason, Wallace, and Walton (1955) reported an outbreak of sepsis due to *Staph. aureus* of phage type 79 which was clearly traced to a surgical assistant. A surgical assistant was also implicated in the outbreak reported by Shooter, Griffiths, Cook, and Williams (1957); of 45 operations in which he took part no less than 24 resulted in sepsis. The staphylococcus responsible was of phage-type 47/53/75/77. In America, Browne, Ryan, Glassow, Martin, and Shouldice (1959) traced infection to a surgeon carrying a staphylococcus of type 81.

The carrier apparently need not necessarily be one of the surgical team who has a hand in the wound in order to cause infection. Penikett, Knox, and Liddell (1958) described infection coming from a scrubbed nurse handling suture material, and Sompolinsky, Hermann, Oeding, and Rippon 1957) thought that nurses performing unscrubbed duties in the theatre were responsible for a number of cases of infection.

Approximately a third of gloves used by surgeons are perforated during the course of an operation and the surprising thing is perhaps that sepsis from staphylococci carried on the surgeons' hands is as infrequent as it appears to be. Presumably, to produce a significant number of cases a surgeon has to have moist hands on which he carries a dangerous staphylococcus. As routine bacteriological examination of all surgeons' hands is impracticable, an individual with these characteristics may cause considerable damage before being detected, especially if adequate records are not kept. Normal scrubbing up with soap and water will not remove all staphylococci from the skin of the true hand carrier. In most cases the regular use of hexachlorophane, either as a fluid preparation or in soap, will abolish staphylococcal carriage or reduce it to a negligible level, and because there is no other single way of guarding against the dangerous hand carrier, the routine use of hexachlorophane appears to be justified.

SEPSIS FROM AIRBORNE STAPHYLOCOCCI Despite the work of Wells and his colleagues in America (Wells, 1934; Wells and Wells, 1936; Hart, 1938a, b), the suggestion that wounds might be infected at operation from the air was not readily accepted in Britain. Bacteriological support came first. Bourdillon and Colebrook (1946) and Bourdillon, McFarlan, and Thomas (1948) examined the bacterial content of air in ward dressing rooms and operating theatres. They showed that in unventilated or poorly ventilated rooms the air might contain many bacteria. These might be derived from reservoirs such as infected dressings or carriers inside the room, or might be sucked into the room by faulty ventilation. When exhaust ventilation was replaced by plenum or positive-pressure ventilation bacterial contamination of the air was strikingly reduced. These views were accepted in theory but for several reasons were usually ignored in practice. One reason was that penicillin had become generally available and, with most staphylococci sensitive, provided an apparent solution to the problems of hospital infection. The solution proved to be only a temporary one. The steady increase in the numbers of penicillin-resistant staphylococci led to renewed interest in operating theatre ventilation, and by this time phage typing was available to assist investigation.

The observation that theatres ventilated with exhaust fans had high air counts was confirmed, but some investigators were able to go further and show that replacement of the original ventilating plant by one which ventilated the theatre under positive pressure not only led to reduced counts of airborne bacteria, but also produced a sharp fall in post-operative wound infection (Blowers *et al.* 1955; Shooter, Taylor, Ellis, and Ross, 1956).

In an operating theatre the ventilation has to perform a number of different tasks. It must provide comfortable working conditions; it has to remove anaesthetic gases, and it has to supply bacteriologically pure air in which the patients' tissues can be safely exposed. These tasks fortunately do not conflict. Published results suggest that the most serious ventilation defect in an operating theatre is the suction of air into the theatre from adjacent wards. It is in wards that resistant staphylococci are bred and ward air may be laden with them. In Britain suction ventilation is still all too common. In many older theatres a single extract fan is installed to remove steam. Some theatres have no artificial ventilation but even in these, because the theatre is usually warmer than adjoining corridors, thermal currents may transfer hospital air to the theatre. And even in new operating theatres the ventilation plant, which on the drawing board was designed to provide positive pressure, may fail to to do so in practice, or may fail to ensure that air flows out from the theatre, both at the bottom and at the tops of doorways, under all circumstances.

There is no difficulty in deciding whether air is being sucked into a theatre. With a plume of smoke from a swab dipped in titanium tetrachloride, air movements can be watched. All new theatres should be tested before they are accepted from the builders. One reason for failure of pressurization has been an attempt on the part of the designer to provide a balanced air input and exhaust; during use the volume of input tends to fall below that of the exhaust, and air is sucked into the theatre in a way not envisaged in the design. To provide reliable positive pressure requires the supply of approximately 12 to 15 changes of air an hour (usually equivalent to about 1,000 cu. ft. per min.). If more than about one third to one half of this air is taken away by an extraction fan, it is unlikely that satisfactory positive pressure will be present. Although it is rare that doors fit so tightly that air will not be able to escape around them, it is probably best to provide planned air outlets with weighted flap valves arranged to open only when there is a pressure difference of 0·1-0·2 in. water gauge between the operating room and the space outside.

Ideally the incoming air should be filtered and humidified, but where for financial reasons this is not possible, or in an interim period while waiting for new building, the risks of suction ventilation may far exceed those of using unfiltered air and it is still desirable to replace existing exhaust fans with input

fans. The size of the risk involved in suction ventilation will plainly depend on the situation of the theatre. If infection were the only consideration, theatres would probably be best built in separate blocks away from wards; temporary hospitals consisting of separate huts would seem to offer a situation in which the validity of this idea could be tested. Conversely, the risk of airborne infection is greatest with theatres on the top floor of high buildings.

In a new theatre, or a reconstruction of an old one, the incoming air should be humidified for comfort and safety and supplied through a filter which will retain 95% of particles down to 5 microns in diameter. Filters of this type will impose no excessive mechanical problems.

Staphylococci may be liberated within the theatre either from the patient or his bedding, or from the staff. Where this can be avoided, it should be, and no patient should come to the theatre with bedding which has been in use in the ward. Carriers present a more difficult problem, as they may shed staphylococci while changing their clothes, while washing, or even when dressed in theatre uniform. Changing and scrubbing-up rooms should be separated from the operating theatre, and the theatre staff should be instructed to plan their work in such a way as to avoid unnecessary movements. However, the surgeon's work is not always easy and it may call for sudden decisions which have to be made in moments of anxiety; over-emphasis on the aseptic precautions may not be in the best interests of the patient.

A further safeguard is provided by the ventilation. At an air-change rate of 12 per hour the concentration of airborne bacteria will on average be only about one third to one half of the concentration that might be found with rates of 2 per hour. This is a further justification for requiring good levels of ventilation, though it is not possible to provide a quantitative estimate of the improvement in infection rates to be expected from any particular improvement in the ventilation rates. There is still some controversy as to the best method of distributing the ventilating air within the theatre but satisfactory results seem to be attainable in practice either by methods designed to give turbulent mixing, or by those giving downward displacement ventilation.

SEPSIS ARISING FROM PATIENT'S OWN ORGANISMS Patients may develop sepsis due to staphylococci which they carried before operation (Williams, Jevons, Shooter, Hunter, Girling, Griffiths, and Taylor, 1959; Weinstein, 1959). Most cases of self-infection probably arise in the ward but the possibility is mentioned here to emphasize particularly the importance of proper skin sterilization.

SEPSIS FROM UNSTERILE INSTRUMENTS AND DRESSINGS Failure to sterilize equipment is a most important cause of clostridial infection and of urinary infection, and will be referred to again. Staphylococci are easily killed by heat, and given satisfactory working conditions and good theatre discipline, it may be doubted if failure to sterilize equipment is more than a very exceptional cause of theatre staphylococcal infection.

In summary, the amount of sepsis originating in the operating theatre is dependent in the first place on the care with which the surgical team carry out aseptic techniques. The breaks in aseptic technique are not always easily recognized by the surgeon. His equipment may, but very rarely, contain staphylococci capable of initiating infection. He or a member of his team may become a hand carrier of an epidemic strain of *Staph. aureus*, or may suffer from actual staphylococcal infection. The patient's own staphylococcus may survive on the skin around the wound or may be transferred to it from unsterilized skin by careless use of towels. And if the ventilation is faulty the most meticulous operative technique may not be enough to prevent sepsis. When confronted with an outbreak of post-operative sepsis it may be worth considering these factors. It may prove that the cause of the outbreak was a specific and recognizable one, rather than, as suggested by the not unusual generalization, that surgeons nowadays brought up with antibiotics are less careful than their predecessors.

CLOSTRIDIAL INFECTIONS

TETANUS Few wounds provide the conditions necessary for clostridia to grow, and this is fortunate, as spores of *Clostridium tetani* are frequently present in hospital dust and may be found in or on many objects (Lowbury and Lilly, 1958a; Report of the Public Health Laboratory Service, 1959). There is very little evidence that tetanus has resulted from direct airborne contamination of wounds: in nearly all reported cases articles contaminated with tetanus spores have come in contact with the wound. Some substances are by their nature and mode of manufacture particularly liable to contamination. Catgut, cellulose wadding, cottonwool, plaster of Paris bandages, and dusting powder have all been incriminated as causes of clinical tetanus, while catgut and dusting powder have been held responsible for tetanus arising at the time of operation.

Catgut is now effectively sterilized during preparation; proper sterilization will also ensure that, even if present, tetanus spores are killed in the other substances. Unsterile and potentially infected articles such as unbleached cottonwool and cardboard should never be brought into the operation theatre.

Patients about to undergo operation on old sites of injury which may still harbour tetanus spores should be protected by immunization. Passive immunization will protect for a time, but as there is rarely any urgency in these cases active immunization is nearly always feasible and much to be preferred.

Tetanus is only rarely seen in this country as a hospital infection but the outcome is so often fatal that a determined and sustained effort must be made to prevent it.

GAS GANGRENE Spores of clostridia capable of causing gas gangrene are even more widely spread in hospital than those of *Cl. tetani* (Lowbury and Lilly, 1958b; Noble, 1961). Many pathologists have had the disturbing experience of isolating *Cl. welchii* from the floors or walls of operating theatres, while clostridia contaminate approximately a third of war wounds and peace-time burns (MacLennan, 1943, 1944; Lowbury and Lilly, 1958b) and it is not unusual to find *Cl. welchii* in post-operative wounds. Despite this, gas gangrene is uncommon. Unlike tetanus, the responsible clostridia have in most cases probably come from the patient himself. This is particularly likely to be so in amputations through the upper part of the thigh.

Prevention of gas gangrene lies again partly in efficient sterilization of articles brought in contact with the patient's wound. But, as there is no form of pre-operative skin sterilization which can be relied on to kill spores, and as the skin within some distance of the anus will probably be contaminated with spores of *Cl. welchii*, high thigh amputation is one of the few operations in which the routine use of large doses of penicillin is justified.

URINARY INFECTION FROM UNSTERILE INSTRUMENTS

Urological surgery is the one field in which proper sterilization of the surgeons' instruments is difficult, and has often been seriously deficient. Some cystoscopes and similar instruments are too delicate to stand sterilization by autoclaving or even by immersion in boiling water. Indeed, it may not be possible, strictly speaking, to "sterilize" such an instrument, but it should be possible to rid it of bacteria pathogenic from the urinary tract. There are now two simple ways of doing this, and of doing it sufficiently quickly to permit of the instrument being used repeatedly throughout an operating session. Francis (1959) described a "pasteurizer" which was simply a water bath thermostatically controlled at 70-75°C. Immersion in this bath for 10 minutes is sufficient to kill vegetative bacteria, and in practice has not been found to damage any but the most delicate instruments.

If a pasteurizer is neither available nor desired, chemical disinfection properly used will kill all vegetative bacteria, other than tubercle bacilli. Methods of chemical disinfection are discussed in the article in this journal by Dr. W. A. Gillespie (page 26).

It is to be hoped that manufacturers will soon produce cystoscopes and their attachments which can be sterilized in an instrument autoclave, or at least in boiling water. In the meanwhile, however, either chemical disinfection or "pasteurization" seems a reasonable compromise. The pasteurizer can also be used for other heat-sensitive equipment like anaesthetic face masks and tubing.

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The surgeon's autoclave

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This difficult, costly, and complex piece of engineering occupies a central place in what is aptly described as the aseptic ritual. The autoclave is intended to guarantee the sterility of articles which have been put into it by exposing them to steam at a greater than atmospheric pressure and therefore at a temperature over 100°C. (212°F.) and usually between 121°C. (250°F.) and 134°C. (274°F.). Within the past five years surgeons' autoclaves in Great Britain have been the subject of critical reports and investigations which clearly revealed that a great many of the autoclaves could not be relied upon to effect sterilization as they were being used (see, for example, Bowie, 1955; Howie and Timbury, 1956; Scott, 1957; Magee and Oakes, 1958; Nuffield Trust Report, 1958; Medical Research Council Report, 1959; and Wells and Whitwell, 1960). From the same work and that of others (see Bowie, 1958a and b; Alder and Gillespie, 1957; Knox and Penikett, 1958; Penikett, Rowe, and Robson, 1958; Rice, 1958; 1960; Medical Research Council Report, 1960) recommendations emerged about better methods for the construction, installation, maintenance, and operation of autoclaves. These communications were received with general interest, and the object of the present paper is to set down what seem to be the main points of all this activity and to emphasize a few principles. If these principles are not understood and acted upon we shall do no more than exchange one form of meaningless ritual for another. The necessity is to devise and ensure the continuous exercise of an effective process.

NEED AN AUTOCLAVE STERILIZE?

Sometimes the question is asked whether autoclaves matter a great deal in the prevention of post-operative wound infection. An answer to this variety of agnosticism can hardly be made in quantitative terms from surgical practice in man as a moment's humane reflection will surely reveal; but the answer is not less emphatic because it cannot be expressed in figures. If an article is labelled or may credibly be presumed to be 'sterile', a surgeon may use it with a clear conscience. But if it may be labelled only 'probably sterile,' dare the surgeon accept it? What will be the effect on his medical and nursing colleagues if he does? Will the sister's grip of the aseptic ritual in her theatre remain effective? We all know of horror stories without a moral: of the autoclave that proved acceptable for seven years although no steam ever entered its chamber, but only its jacket, until at last this engineering defect was discovered during the investigation of an outbreak of post-operative tetanus. What do we rate as significant: the seven years in a paradise of ignorance or the one catastrophe? I know of a sterilizer which never revealed its defect until, challenged by unbleached cotton wool, which was suddenly introduced as a war economy measure, it demonstrably failed to kill the tetanus spores in the cotton wool in its lower third. Moreover, on further tests, it also failed to kill staphylococci in the same area. These facts about this particular sterilizer were discovered only after the majority of the patients of one morning's list had developed post-operative tetanus. Its discharge channel was ineffective. I know of another sterilizer which did not produce any disturbance in the minds of those who relied on it although dressings that came out of it were contaminated with a variety of organisms during the drying process. The contaminants found were mostly aerobic Gram-positive sporing bacilli and micrococci but at different times they also included *Clostridium welchii* and *Staphylococcus aureus*, organisms not present in the dressings before sterilization, so that a witty medical student said of the apparatus: 'Not perhaps much of a sterilizer—but a fertilizer!' Such tales are typical of those that abound wherever people meet who are concerned with autoclaves; and they are not the mere imaginings of enthusiasts, as is confirmed by the Nuffield Report (1958) and by the writer of 'Any Old Iron' (1959). An inefficient autoclave may not reveal its failure in terms which cause clinical misgiving for many years. In that sense the evidence suggests that autoclaves may not be very important quantitatively as direct causes of hospital sepsis. In outbreaks of post-operative tetanus and gas gangrene, they should obviously receive more critical study than they have often had during the investigation of some such reported episodes. Because they kill one packet of test spores, there is

no guarantee that they can do so in every kind of package in any part of the load or that they do not recontaminate sterilized dressings during the drying process, and what more efficient way is there of concentrating the bacterial contamination of hospital air in a situation where it will really matter than of drawing it through a defective filter into a set of surgical dressings? And if, as often, the defective sterilizer also soaks the dressings and this has upset the surgeon, will not the sterilizer attendant make certain that the airing process at least is thoroughly done? I know of sterilizers where the drying process habitually lasted an hour or longer, and of one in particular where the process of drying was completed by placing the autoclaved drums, with open ports, on top of a radiator. The surgeon's reaction to being given a wet dressing was more predictable than the patients' reaction to being treated with one that was not sterile. Lister, whose earliest concern was mainly with airborne microorganisms, much later acknowledged that he was then more worried by 'grosser forms of septic mischief.' Those who so naïvely presumed upon their understanding of the realities of hospital bacteriology as almost wholly to supplant Lister's antiseptic methods by their aseptic ritual were surely an unwitting source of septic mischief. We cannot measure how much. But need we? As the advisers of surgeons surely we can stand on the doctrine that asepsis is meaningless ritual, charged with hidden errors and unmeasured risks, unless all that is labelled and regarded as sterile carries a valid guarantee that it is indeed free from microorganisms. It is true that some apparatus need not be sterile, cytoscopes, for example (Francis, 1959), and deliberate decisions merely to decontaminate certain articles may be accepted by those responsible provided they know exactly what they are doing. But for all that, the great majority of articles used in surgery can and should be sterile; and the instrument most relied upon to effect this sterilization is the autoclave. Therefore we must see to it that our surgeons' autoclaves are effective in their design and use.

WHO IS RESPONSIBLE?

The surgeon is a busy man, skilled in his particular and difficult duties, but he has not the outlook or experience of autoclaves possessed by a mechanical engineer, a pharmacist, or a bacteriologist. Reasonably, he looks for someone to see to the sterilization of his dressings and instruments. Often the theatre sister accepts this responsibility. But what does she know from her training of the working and maintenance of all the different varieties and makes of autoclaves? As much, probably, as a railway guard knows about the locomotive that pulls his train. Sister will faithfully do what she has been taught, but autoclaves differ from each other in important details and sisters move around. Theatre superintendents, hospital porters, and relatively junior nurses may also operate autoclaves after receiving only the minimum of instruction. Often, as accidents to such operators have revealed, they know very little about the true nature of the engineering considerations that govern the correct use of steam under pressure. If the engineer, usually an overworked person, is called to a faulty sterilizer he may well be content to repair obvious defects and leave it at that. If he has to maintain and service in one hospital several different makes of autoclave he will need a great variety of spare parts; and if his well meant advice is not readily understood or willingly accepted he may be pardoned for feeling that the calorifiers for heating the hospital deserve more of his attention and skill than the autoclaves; in any case the end product of the autoclaves is not his direct concern and he is unlikely to be unduly worried about their deficiencies unless someone else shows real concern. If an autoclave falls under suspicion, the bacteriologist is likely to be called in because he is supposed to know about spore tests and because his work depends on efficiently functioning autoclaves. Unfortunately, the bacteriologist's own working experience of autoclaves may not guide him to give the right advice about the surgeons' autoclaves.

Bacteriologists rely on their autoclaves mainly to sterilize media, all of which are fluid during sterilization, and instruments. These are easily sterilized because the nature of the load offers no serious obstruction to attainment of the steam temperature throughout the autoclave and the articles within it. The simple pressure-cooker type of autoclave is readily able to meet the bacteriologist's needs, and the danger is that he may fail to realize how different his instrument is from that required to steam and dry a load of packaged fabrics and other goods required for use in an operating theatre or ward. Until recent reports became required reading for hospital bacteriologists, many of them revealed their unawareness of the true mode of action of the surgical autoclave both by the advice they offered and by the over-simple and essentially unsatisfactory tests which they advised to check the functioning of surgeons' autoclaves.

Consequently, we may be forced to conclude that one of the principal needs for efficient autoclaving is that some member of the medical staff both educates himself about autoclaves and undertakes the responsibility of being answerable to his medical colleagues on two issues: (1) that adequate methods and apparatus are employed and are under the day-to-day care of a person of sufficient professional

standing to guarantee the faithful and exact performance of what is laid down; and (2) that regular checks of various kinds are applied, recorded, inspected, understood, and acted upon. Both the Nuffield Report (1958) and the first M.R.C. Report (1959) laid great emphasis on the need to define responsibility in this way.

CENTRALIZED AUTOCLAVING

Obviously both the medical man accepting such responsibilities and the day-to-day superviser can discharge their tasks only if the autoclaves in their charge are not too widely dispersed in many places. In practice this means that operating theatres will rely upon a central source to supply their sterile packaged goods but will have their own autoclaves for unwrapped instruments and bowls. Instruments and bowls are easily and quickly sterilized in a very simple type of autoclave (M.R.C. Reports, 1959 and 1960), which may yet be further simplified by the outcome of experiments still in progress. Such instruments may confidently be left to sisters and nurses, especially if their practical training and examination can be made to include the working of this particular piece of apparatus. Bottled fluids will probably remain in the charge of the pharmacist, and here again there is no reason to anticipate difficulty, both because the problems are fundamentally straightforward and because the training and examination of pharmacists lays considerable emphasis on the correct performance of sterilizing techniques. Current experiments with new designs of autoclaves for bottled fluids may be expected still further to increase the security of this particular sterilization process. Surgical dressings and other packaged goods, including rubber gloves, thus become the main problem of the centralized autoclaving unit, and it is to this that the rest of this article will be directed. The problems of centralization and of guaranteed performance have been simplified in the last few years out of all recognition by the introduction of high-vacuum autoclaves with automatic controls. The enhanced capacity of such apparatus was clearly demonstrated by Wells and Whitwell (1960).

REQUIREMENTS FOR SURE AND SAFE AUTOCLAVING

A GOOD STEAM SUPPLY To guarantee the rapid killing of resistant spores, especially in textiles, it is necessary to have a reliable supply of steam under increased pressure at a temperature of from 121 to 134°C. (250-274°F.). If this steam is what is technically known to engineers as 'just dry' it will condense and penetrate rapidly with lethal effect into the substance of even the most resistant spores and it will not do more than merely dampen the dressings. However the steam is produced, direct from the mains or generated in apparatus near to or actually part of the autoclave, it must be dry and without more than 5°C. of superheat. Commonly, from inadequate lagging and trapping, hospital mains steam supplied to autoclaves carries more than 10% of its weight made up of liquid water and such steam is unacceptable. Its improvement is essentially an engineering problem. Fitting adequate separators near to the sterilizer is of great value, but it must be emphasized that a reliable supply of steam is the first necessity for efficient autoclaving, of which the criteria are the regular and rapid production of dressings that are both sterile and dry.

REMOVAL OF AIR All parts of a load of textiles to be sterilized must be freed of air so that the load may be permeated by the steam. Autoclaves of the pressure cooker type make no provision for the removal of air during sterilization; hence they are unable to sterilize textiles. Until comparatively recently, this important matter was not widely understood by many who operated sterilizers in this country, and some do not yet appear to have grasped the point. There is little to be gained from the commonly used practice of employing steam-ejector mechanisms to draw a vacuum, or even two vacuums, of the order of 15 in. Hg or less. These low-order vacuums waste a great deal of steam and time and add to the damage to sensitive fabrics, such as rubber gloves. They remove some free air from between packages but they do not guarantee the almost instantaneous penetration of steam into all parts of the load of dressings. Although low-order vacuums could clearly help in marginal conditions, they are not to be recommended in practice. Air should be removed from the dressings either by its gradual displacement by steam or by drawing a high-order vacuum, that is, a vacuum greater than 29·2 in. Hg. The small amount of air remaining in a load subjected to such a vacuum must be displaced by steam during sterilization to ensure that all parts of the textiles are sterilized. Simple displacement (also known as gravity-displacement or downward-displacement) sterilizers can be very efficient, but only if they are operated with a degree of skill and care which cannot by any evidence of recent experience be ascribed to the general group of people entrusted in this country with the operation of surgeons' autoclaves. For success, a steam supply of the right quality (see above) must be used upon a load lightly packed and properly positioned in a sterilizer fitted with a reliable automatic discharge mechanism with an adequate near-to-steam trap. The quality of the

steam is checked, to some extent at least, by ascertaining if a thermometer placed in the discharge line gives a temperature reading corresponding to the pressure.

CORRECT LOADING AND EXPOSURE The loading of autoclaves is often done in such a way as to guarantee that, by steam displacement alone, all the air cannot be removed. Packs are more easily penetrated than drums, but they call for more careful storage. Cardboard boxes have certain attractions; they appear to be more easily penetrated by steam in high-vacuum autoclaves than are drums of the kind generally employed in hospitals; conversely they are rather less satisfactory in this respect in simple displacement autoclaves. Packs have much to commend them if the needs for their safe storage can be met (see M.R.C. Report, 1959): they have to be protected from dust, damp, and insects. Specially made metal caskets with built-in filters have a high initial cost but many advantages which, in the long run, may prove them to be the best form of container. Very large packs or drums or tightly packed loads will defeat steam penetration unless a vacuum of the order of 29·2 in. Hg can be drawn before steam is admitted. It is hardly realized how difficult it is to get steam through the loads commonly put into sterilizers. This, perhaps more than anything else, is where imperfectly trained and inexperienced operators fail in their understanding. High-vacuum sterilizers are justified because they overcome this problem and enable so great a shortening of the sterilizing cycle that few autoclaves are needed (Wells and Whitwell, 1960) and the temptations to overpacking are reduced. Likewise if fewer autoclaves are needed it is easier to centralize them under the control of a relatively small number of more skilled attendants. Those who have to deal with old sterilizers need not necessarily regard their task as hopeless. Some old sterilizers can be put into a state of reasonable efficiency (for details see Scott, 1957) without too much trouble and may be worked satisfactorily if enough skill can be devoted to them. It is with such autoclaves that care in packing and positioning is specially important. Without this, such sterilizers offer a poor guarantee of efficiency. Some downward-displacement autoclaves may be successfully upgraded to high-vacuum status either by local engineering modifications (Magee and Oakes, 1958) or by fitting conversion units which also provide automatic control (Alder and Gillespie, 1959). Such upgradings and conversions should not be embarked upon without first consulting the makers and the insurers of the sterilizer concerned.

Times of exposure for a properly packed load have been much discussed and debated. What will serve as a minimum, *e.g.*, for unwrapped instruments, will not be enough for fabrics in packs or dressing drums. The M.R.C. Report (1959), which gave a reasoned statement, recommend the following times and temperatures as a minimum exposure after the steam had reached the correct temperature for the pressure employed:

3 min. at 134°C. (30 lb. per sq. in.)
10 min. at 126°C. (20 lb. per sq. in.)
15 min. at 121°C. (15 lb. per sq. in.)

This short period of 3 min. at 134°C. is applicable only to high-vacuum, high-pressure sterilizers which are efficiently maintained and serviced and so fully controlled that any failure of the mechanics of sterilization is made clear by appropriate warnings to the operator. These sterilizers obviously allow a very short time margin to cover any error, and full attainment of the right conditions at every stage must therefore be automatically monitored and all failures made evident. If downward displacement is used for removal of air, and if the fabrics are correctly packed and loaded into a fully efficient sterilizer, the M.R.C. Report (1959) recommended and gave reasons for the following for temperatures of 121 and 126°C.

	min. at 121°C. (15 *lb. per sq. in.*)	*min. at* 126°C. (20 *lb. per sq. in.*)
Instruments and utensils	15	10
Fabric packs	30	20
Dressing drums	45	30

With high-vacuum equipment used under ideal conditions the complete cycle may be accomplished in 20 minutes or less. Obviously each sterilizer manufacturer ought to be consulted and the actual performance of any new sterilizer installed should be critically checked by a competent engineer and bacteriologist to ensure that whatever is laid down for the particular sterilizer is correctly decided.

Gloves and Glove Powder One point that needs to be emphasized is that gloves should not be sterilized at low pressures, for example, at 10, 7, or even 5 lb. per square inch. If properly packed and not over-dried, gloves may be sterilized in the same way as other materials without being damaged. Imperfectly sterilized gloves are a serious danger especially if, as all too often, they are treated with glove powder, which has not been correctly sterilized. If glass jars of glove powder with screwed-down lids are put into autoclaves, it may be asserted that sterilization of the contents is impossible. Glove powder in small packets of guaranteed sterility is available commercially, or the powder may be spread out flat on trays and baked in a hot-air oven at 160°C. for one hour and carefully filled, with sterile precautions, into small packets whose outside

may be sterilized in the autoclave. Steam does not sterilize talcum powder because this substance is unwettable.

DRYING WITHOUT RECONTAMINATION Autoclaved dressings are often dried by drawing warm filtered air through them for periods that appear to range from 18 minutes to several hours. With the filtration methods hitherto commonly employed, or even in the absence of any filtering mechanism, which is not a rare situation, it is difficult or impossible to ensure the sterility of the warmed air (around 56°C.) which is drawn into the sterilizer and may be passed without further ado directly through the open ports of unlined drums straight into the dressings. Various types of filters made of cottonwool or other fabrics have served well enough if conscientiously changed and correctly fitted. Rice (1960) designed a filtration set-up for autoclaves which greatly improved the prospects for efficient use of cottonwool filters. Work recently reported to the M.R.C. Working Party (M.R.C. Report, 1960) now suggests that glass fibre woven into sheet form of paper thickness offers the best prospects of a reliable filter which will stand up to autoclave conditions and have a working life of at least 12 months. The glass-fibre filter requires the support of metal grills to prevent distortion and it must be set in a well-made metal canister. A critical orifice is required to ensure uniformity of flow and prevent excessive strain on the filter. Apparatus of this kind is now available from at least two manufacturers in this country.[1] Such filters should be highly efficient with a methylene-blue penetration of not more than 0·003%.

An important aspect of the problem of drying without recontamination is to make sure that the dressings are dry at the outset, to use dry steam, and to draw a vacuum of 28 in. Hg after sterilization. If this vacuum is drawn (it need not be held for even five minutes) and if filtered air is then used to break the vacuum, nothing more should be required. If the dressings are still wet after such treatment, an engineering fault needs to be found and treated.

TESTING AUTOCLAVES

Full comprehension of all the details of the principles and practice of pressure-steam sterilization is not normally to be expected of the bacteriologist, who will be well advised, before tackling any autoclave about whose performance he is consulted, to insist that it should first be checked by an engineer who knows the apparatus and its function and can demonstrate the steam circuit of the particular autoclave. Such details of this kind as a bacteriologist may require to know are emphasized and summarized in the two M.R.C. Reports (1959; 1960). When he is satisfied that the apparatus is accepted as in working order by the engineer, the bacteriologist should inspect the recent records of temperature and pressure which ought to be made for every sterilizing cycle and kept for inspection. The regular inspection of such records by someone who knows what they mean is itself probably the best single test of any autoclave. Too often such records are made but not examined by anyone who feels responsible for action if they reveal some failure of the autoclave. It would be a good custom if it came to be that every record were signed by a responsible member of the medical staff as well as by the sterilization attendant. A properly designed and correctly instrumented autoclave thus tests itself and makes its own record. In the past, no doubt, the form of such records has often been very hard to understand and their form has often been meaningless because they did not always record the steam temperature independently of the pressure. A temperature recording instrument with its sensitive end in the discharge line is essential, as well as a direct easily read thermometer. Recordings made from a dummy load in the lowest part of the sterilizer are an even better indication of performance. For special checks of the temperature attained in different parts of the sterilizer, direct-reading portable thermocouples are available. This type of apparatus enormously enhances the ease with which faults may be quickly detected and localized.

Routine bacteriological checks with spores of various kinds have been the subject of much confused thinking by bacteriologists and others. In many cases these tests have become as meaningless a ritual as other forms of inadequate operation of autoclaves. Such tests are no substitute for correct records of autoclave performance and scarcely necessary if these are properly kept and studied. As Kelsey (1958) established, many unsuitable spores have been employed, and intelligent use of chemical indicators has many advantages. Brown and Ridout (1960) made a careful and critical study of the ways of using such indicators and showed both their possibilities and limitations. In the past, it is clear that many tests, both with spores and chemical indicators, have been little more than an act of self-deception and have been welcomed mainly because they offered an impressive and apparently simple alternative to making sure that the autoclave was really able to do what was required of it. In checking new sterilizing methods and types of sterilizer, in making sure that recommended times and temperatures are sufficient, in devising new types of packaging, and in reassuring those conditioned to accepting only bacteriological

[1] (1) E. N. Mackley & Co., Hawks Road, Gateshead 8, Co. Durham.
(2) Vokes Ltd., Henley Park, Guildford, Surrey.

checks, well-standardized preparations of bacterial spores have certainly a place. Kelsey (1961) has produced valuable information about the effective use and standardization of *Bacillus stearothermophilus* spores for this kind of work. Suitable spore preparations, if strategically placed in various critical positions in a sterilizer, give useful additional evidence and corroboration of the results of other tests; but nothing valuable is established by culturing routinely a single preparation of spores of uncertain standard placed wherever the sterilizer attendant cares to put it. A drum containing a few sterilized swabs placed near the air filter inlet may usefully be employed to check the efficiency of the air filter (Howie and Timbury, 1956). In spite of these generally critical observations, a few well-sited Browne's tubes (type II for high-vacuum, high-pressure sterilizers; type I for downward-displacement sterilizers) often give early warning to a conscientious theatre sister that her dressings have not been adequately exposed and that the autoclave needs a thorough investigation. The bacteriologist with his spores should come in only to test new methods and as a reassurance when required, for example, after an autoclave has been found inefficient and has been repaired. In short, it is not by some simple laboratory test that the bacteriologist will best serve the cause of efficient autoclaving and sterilization processes generally. He can discharge his professional duty in this matter, however, if he keeps himself up to date with new work on the subject, assesses it critically in relation to the problems of his own clinical colleagues, accepts medical responsibility for the work of the sterilizing department if this is so arranged as to make medical supervision a possible reality, and conducts a continuous process of education both for those who operate autoclaves and for those whose work depends on the guarantee of sterility which these instruments are intended to provide. If he can do all this and remain a good-humoured colleague he will indeed be entitled to regard himself as a worthy follower in the succession which includes Lister and Colebrook, in honour of whose work this article was written.

CONCLUSIONS

This article reviews much of the recent work which has shown that surgeons' autoclaves have been too readily accepted as reliable and efficient.

The main principles governing the successful operation of autoclaves are briefly stated with references to published works in which the necessary matters of detail are fully set forth.

As new types of autoclaves come into use, and while older autoclaves are being upgraded pending re-equipment and the general establishment of centralized autoclaving, bacteriologists have an exacting duty to perform in gaining a full understanding of how surgeons' autoclaves work and of exercising a general function of education and supervision.

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Radiation sterilization

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SYNOPSIS The sterilization by radiation of disposable medical items has been investigated. Various vegetative and spore-bearing bacteria have been irradiated and results indicate that 2·5 Mrad can be recommended to give a high degree of sterility. Spores of *B. pumilus* (E.601) showed such resistance that it might be a suitable test organism for determining the efficiency of a radiation treatment. A high standard of packaging must be demanded for pre-sterilized products.

Several pre-sterilized disposable medical items, such as plastic hypodermic syringes and catheters, are now available in Great Britain and many more are likely to be marketed in the near future. It is often necessary for economic reasons to manufacture some of these items from cheap thermoplastic materials so that a 'cold' method of sterilization must be used for treating appliances made in this way.

Two main sterilizing agents are available to the manufacturer, ethylene oxide and ionizing radiations, and the final choice of technique is determined by the particular application. Sterilization by radiation is achieved in practice by the use of either high-energy electrons from a machine, such as a linear accelerator, or gamma radiation from an isotope source such as cobalt 60. Both types of ionizing radiation have been used commercially for sterilization, and they have similar bactericidal properties, but technically they differ in the characteristics of dose rate and degree of penetration. Absorbable surgical sutures sterilized by high-energy electrons are marketed in the United States by Ethicon Incorporated (Artandi and Van Winkle, 1959) and sutures are also being sterilized by gamma radiation in Melbourne, Australia, at a large cobalt 60 installation which is basically intended for treating imported animal fibres contaminated by anthrax. The package irradiation plant at the Wantage Research Laboratory is now being used to sterilize large quantities of medical products intended for industry and hospitals for marketing and clinical trials. Several items sterilized at this plant are now available on a limited scale in this country. Radiation sterilization is not, however, the answer to all sterilization problems because certain products are adversely affected by the treatment. Nevertheless, experience in the past few years has shown that it is a very useful addition to the sterilization techniques available, in particular to the manufacturers of medical and pharmaceutical products. The current status of radiation sterilization has recently been reviewed by Powell and Bridges (1960).

The purpose of this paper is to report the bacteriological results and some practical experiences of two laboratories concerned with the application of radiation sterilization. Part I deals with application to disposable balloon catheters and Part II concerns treatment of disposable plastic hypodermic syringes.

STERILIZATION OF CATHETERS

METHOD The following spore-bearing organisms were used in the trials and were prepared as previously described (Darmady, Hughes, and Jones, 1958): *B. stearothermophilus*, N.C.I.B. No. 8157, *Cl. tetani*, N.C.T.C. No. 5411, and dried garden soil.

From microscope slides small rectangular glass strips about 26 × 5 mm. were cut. On to these were placed either two drops of a broth suspension of *B. stearothermophilus* spores, two drops of a Robertson's meat broth culture of *Cl. tetani* containing at least 50% spores, or two drops of a suspension of dried soil in serum. The slides were dried in air at a temperature of 60°C. for 30 minutes and placed in lightly plugged 3 in. × ¾ in. pyrex tubes. The material was sent to Wantage. It was irradiated by a cobalt 60 source and on return to Portsmouth the organisms were tested for viability by the following methods:

B. stearothermophilus, by dropping the slide into a tube of broth and incubating at 60°C. for 48 hours.

Cl. tetani, by dropping the slide into a tube of Robertson's meat medium and incubating at 37°C. for seven days, and then subculturing on to blood agar under anaerobic conditions, readings being made at 48 hours and seven days.

Dried soil, by dropping half the total number of slides into nutrient broth and half into Robertson's meat

medium. After seven days' incubation subcultures were made on to blood agar aerobically from the former and anaerobically from the latter. Readings were again made at 48 hours and seven days.

On return from Wantage about 10% of the slides from each experiment were stored aseptically in daylight at room temperature for 28 days before being cultured to guard against the possibility of the organisms regenerating.

In all cases control slides were kept in the laboratory at room temperature until the test slides had been received back from Wantage. They were then treated in a similar fashion.

Approximately 100 slides of each organism were subjected to each dose of radiation and the results are shown in Table II.

Several catheters were infected in a similar way with *Cl. tetani* and dried soil and irradiated at various dose levels. The numbers tested are shown in Table I. They were tested for sterility using the techniques described above.

TABLE I

RESULTS OF STERILITY TESTS ON INFECTED CATHETERS

	Numbers of Catheters Examined		
Organisms Tested	2·0 *Mrad*	2·5 *Mrad*	3·0 *Mrad*
Cl. tetani	38	6	2
Dried soil	6	6	2

RESULTS Growth occurred in all controls retained in the laboratory. None of the organisms tested survived a dose of 2·0 Mrad and there was no evidence of photoregeneration after exposure to daylight at room temperature for 28 days. *Cl. tetani* appeared to be the most resistant of the organisms tested. All infected catheters were found to be sterile at all dose levels.

TABLE II

RESULTS OF STERILITY TESTS ON IRRADIATED SAMPLES OF GLASS SLIDES

		Percentage Sterile		
Organisms Tested		1·0 *Mrad*	1·5 *Mrad*	2·0 *Mrad*
B. stearothermophilus	(a)	90 (90)	100 (80)	100 (90)
	(b)	90 (10)	100 (10)	100 (10)
Cl. tetani	(a)	83 (80)	92 (90)	100 (90)
	(b)	100 (10)	90 (10)	100 (10)
Dried soil	(a)	95 (90)	100 (90)	100 (94)
	(b)	100 (10)	100 (10)	100 (10)

Figures in brackets denote number of slides tested; (a) on return from Wantage and (b) after photoregeneration test.

STERILIZATION OF PLASTIC HYPODERMIC SYRINGES

METHOD Twelve organisms, six vegetative and six spore-bearing, were used for this investigation.

The vegetative organisms were grown in nutrient or glucose nutrient broth at optimum temperature for 18 hours. The spore-bearers, after specific spore preparation, were used as suspensions in sterile distilled water.

PREPARATION OF SPORES For *B. subtilis* and *B. pumilus*, an overnight nutrient broth culture was flooded on the dried surface of a nutrient agar plate, incubated 18 hours at 37°C., then left for two days at room temperature.

For *B. globigii*, an overnight glucose nutrient broth culture was flooded on the dried surface of glucose plus 1% activated charcoal agar plate, incubated 18 hours at 37°C., then left for four days at room temperature.

For *B. stearothermophilus*, as recommended by Alder and Gillespie (1957).

For *Cl. welchii*, as described by Ellner (1956).

For *Cl. tetani*, a Robertson's meat broth culture was incubated for three days at 37°C., then left at room temperature until good sporulation occurred.

After harvesting and washing with sterile distilled water, the spore suspensions were heated at 80°C. for 10 minutes except for *B. stearothermophilus* which was heated in a boiling water bath for 15 minutes.

These broth or water suspensions were used to inoculate sterile filter-paper discs by placing one drop (0·02 ml.) from a calibrated Pasteur pipette on to each disc. After drying at 55°C. for 30 minutes, each disc was placed aseptically in a small polystyrene container with a polyethylene cap and sealed in a polyethylene envelope, simulating materials used in the manufacture of plastic syringes. The packages were irradiated in the spent fuel rod assembly at A.E.R.E., Harwell.

For each of the 12 organisms tested, 100 inoculated discs were irradiated at each of four dose levels and 10 discs were kept as controls.

A surface plate count was made of one drop of each bacterial suspension, to ascertain the size of the disc inoculum.

After irradiation, the polyethylene bags and polystyrene containers were opened and each disc transferred aseptically to the appropriate liquid medium which we incubated at optimum temperature for 21 days. For th viability tests, nutrient or glucose nutrient broth was us.. for the vegetative organisms. For *B. subtilis* and *B. pumilus* nutrient broth, for *B. globigii* glucose nutrient broth, for *B. stearothermophilus* dextrose-tryptone broth, for *Cl. welchii* Brewer's medium, and for *Cl. tetani* Robertson's cooked meat medium were used. All bottles of media containing discs were examined after 24 hours', three days', and 21 days' incubation, when those showing growth were checked by subculturing on 10% horse blood agar. All bottles of Brewer's and Robertson's cooked meat media were subcultured on 10% horse blood agar at the end of the incubation period of 21 days. The results of this test are shown in Table III.

After this investigation a small preliminary experiment was carried out on commercially packed plastic hypodermic syringes. Fifty assembled syringes packed in a single plastic envelope were received from the manufacturers. Forty of these were irradiated at 2·5 Mrad and 10 were left unirradiated for use as controls. Using aseptic precautions to remove them from the envelope, each barrel and plunger was dropped separately into 100 ml. quantities of sterile nutrient broth and incubated at 37°C. for 21 days. After incubation, the presence of turbidity in the broth was taken as evidence of growth.

TABLE III

RESULTS OF STERILITY TESTS ON IRRADIATED SAMPLES OF INOCULATED PAPER DISCS IN PLASTIC CONTAINERS

Organisms Tested	*Number of Organisms per Disc*	*Percentage Sterile after 21 Days' Incubation*				
		Control	1·0 *Mrad*	1·5 *Mrad*	2·0 *Mrad*	2·5 *Mrad*
Pseudomonas pyocyanea (NMH)	$2{\cdot}6 \times 10^7$	0	100	100	100	100
Staphylococcus aureus (NMH)	$1{\cdot}1 \times 10^7$	0	100	100	100	100
Streptococcus viridans (NMH)	$1{\cdot}2 \times 10^7$	0	100	100	100	100
Streptococcus faecalis (NMH)	$1{\cdot}5 \times 10^7$	0	100	100	100	100
Escherichia coli (NMH)	$8{\cdot}7 \times 10^6$	0	100	100	100	100
Micrococcus cryophilus	$4{\cdot}2 \times 10^4$	0	100	100	100	100
Bacillus subtilis (NMH)	$8{\cdot}1 \times 10^4$	0	29	100	100	100
Bacillus globigii	$1{\cdot}7 \times 10^6$	0	100	100	100	100
Bacillus pumilus (E.601)	$5{\cdot}8 \times 10^7$	0	72	97	94	100
Bacillus stearothermophilus (8157)	$1{\cdot}6 \times 10^5$	0	90	100	100	100
Clostridium welchii (8797)	$2{\cdot}3 \times 10^5$	0	0	68	100	100
Clostridium tetani (5411)	$3{\cdot}2 \times 10^5$	0	100	100	100	100

One hundred paper discs per organism were irradiated at each dose level. (NMH) isolated at North Middlesex Hospital.

RESULTS. Of the six vegetative organisms tested all the inoculated discs were sterile at each of the four dose levels used. With the six spore preparations tested a greater resistance to radiation was shown by survival at the lower dose levels. Of the aerobic spores, those of *B. pumilus* were the most resistant, a few surviving after 2·0 Mrad irradiation. *Cl. welchii* was shown to be the more resistant of the two anaerobic spores tested, some surviving after 1·5 Mrad irradiation.

Of the commercially packed syringes examined after irradiation growth occurred only in one tube of broth containing a plunger. This was identified as a pure culture of a micrococcus. All the unirradiated control syringes produced heavy turbidity in broth from which spore-bearing organisms were isolated. It is suggested that the isolation of the micrococcus from one irradiated sample might indicate contamination during transfer of the plunger to the broth.

DISCUSSION

In a previous communication (Darmady, Hughes, Jones, and Verdon, 1959) it was shown that between 9% and 50% of equipment used in hospital wards and stated to be sterile was, in fact, contaminated. At that time the alleged sterility of catheters was not investigated but there was no reason to expect that the results would be any better. In order to confirm this we have recently carried out an examination of catheters prepared by traditional ward procedures and have found that out of 10 examined six were sterile and four were infected. Micrococci were isolated from all four of the infected catheters and in addition coliform bacilli were isolated from one.

It is recognized that steam under pressure will sterilize providing the catheters are properly cleaned. In our experience this method is time consuming, and repeated heat sterilizing also causes deterioration of the rubber. In the Portsmouth laboratory other methods of treating catheters have been tried, such as formalin vapour, ethylene oxide, and soaking in various disinfectant fluids, but these have proved to be unreliable and will be the subject of a further communication. As an alternative to these methods, it seemed that radiation sterilization should be investigated for such items as catheters and disposable plastic syringes.

The results of this investigation indicate that spore-bearing organisms are more resistant to radiation than the vegetative forms, and of the organisms tested, the spores of *B. pumilus* (E.601) appear to be the most resistant. It is not considered that the higher number of spores per disc used for this organism (see Table III) could completely account for this apparent resistance as compared with the other spore-bearers. This strain has previously been examined for radiation resistance by Pepper, Buffa, and Chandler (1956) and it appears to be a possible test organism for assessing the efficiency of radiation sterilization. It requires no special spore preparation as it sporulates readily on nutrient agar. A disadvantage may be that it is difficult to differentiate between this strain and normal laboratory contaminants. Nevertheless, other bacilli used as test organisms, for example, *B. globigii* for ethylene oxide and *B. stearothermophilus* for moist heat, are not suitable for use with radiation sterilization because of their lower radiation resistance, and the clostridia, although showing some resistance to radiation, have such cultural requirements that they are unsuitable as routine test organisms.

It is apparent from the examinations of the two laboratories that there is some slight difference in the results that were obtained with *Cl. tetani*. This

difference might be due to variations in the preparation of the spores, initial concentration of spores used, the use of different supporting media, *i.e.*, glass slides and paper discs, or even due to the fact that in one laboratory the spores were not washed, so that a certain amount of broth might adhere to the spore coat, providing some degree of protection. It is not certain which of these variables can account for this difference, but these are being further investigated. Nevertheless, the results indicate that a dose of 2·5 Mrad is adequate for providing complete safety and is to be recommended. This agrees with the conclusions of other workers (Artandi and Van Winkle, 1959; Report of Association of British Pharmaceutical Industry, 1960; Oliver and Tomlinson, in press).

Recently, such disposable items as catheters, needles, and plastic syringes, which have been sterilized by gamma radiation at 2·5 Mrad, have become commercially available in this country. Thirty of these catheters, which were doubly packed in polyethylene envelopes, have been tested and all were sterile.

Although this method allows considerable choice of packaging materials, it is not enough to choose a material that is strong and is impermeable to microorganisms. The standard of sealing the package must be high, and methods for aseptically removing the sterile article from the package must, wherever possible, be incorporated into the package design. As an example of inadequate sealing, we have examined over 1,600 presterilized disposable needles which were packed in polyethylene envelopes, and found that 18% of these had defective seals. Similarly, we have tested the ability to remove the needle from the container and found that out of two series of needles with 400 needles in each series from two different manufacturers, 7% and 1·5% were contaminated. In each case the method of opening was to burst the hub of the needle through the packaging material, and it is clear that the needle could not be transferred to the syringe without the risk of contamination. As a control, 100 needles sterilized by dry heat in a convenient container were found to be sterile when removed.

From our observations, therefore, it is important to realize that maintenance of sterility must be beyond reproach, and it is known that many manufacturers are giving considerable thought to improving standards of packaging.

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Sterilization by ethylene oxide

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Although sterilization by steam under pressure and by hot air are procedures which are now well understood and efficient when properly conducted, there is an increasing number of objects in medical or surgical use which are too heat labile to be so treated. It is for such items as plastic catheters, disposable plastic syringes, and prostheses that ethylene oxide has been proposed as a convenient sterilizing agent. This has been used for years for disinfesting spices and for preventing bacterial spoilage in foodstuffs, but it was only after the publications of Phillips and Kaye (1949) that the possibilities of ethylene oxide as a sterilizing agent began to be appreciated.

Ethylene oxide is a colourless liquid with a boiling point of 10·7°C. At temperatures above this it is a gas with a characteristic ethereal smell. It forms an explosive mixture when more than 3% of it is present in air and it is moderately toxic; its toxicity may be compared with that of ammonia. It is very soluble in water and also dissolves in rubber and many plastics. Although the gas forms explosive mixtures with air, if it is mixed with carbon dioxide or with freons in proportions of not more than 12% by weight the mixture is not inflammable.

Chemically ethylene oxide is a powerful alkylating agent and as such is much used in industry. Its bactericidal effect is probably due to this action: it is lethal to bacteria and to viruses. It is of interest that although vegetative organisms are more easily killed than are spores the difference in resistance is not very great compared with that found in sterilization by hot air or steam. The organism commonly used to test sterilization by ethylene oxide is *Bacillus subtilis. var. globigii* but *Staphylococcus aureus* can be almost as resistant. The gas penetrates readily and will pass into the lumen of small tubes, and through fabrics, paper, and many, but not all, plastics.

Unlike sterilization by steam or hot air, where it is comparatively easy to lay down a safe time and temperature to guarantee a sterile load, the use of ethylene oxide is more complex because a greater number of variables need to be considered. These were defined by Phillips and Kaye (1949). As would be expected sterilization depends on time and concentration of the gas: when the concentration is doubled the exposure time can be halved. The temperature of the reaction is important and the exposure time is halved for every rise of 17°C. (30°F.). It was also shown that the relative humidity can be critical, the optimum being between 20% and 40%. It would seem, however, that the local humidity of the bacteria to be killed is more important than that of the chamber in which they are exposed. Bacteria which have been desiccated are very difficult to kill. Not only must the ambient humidity be correct but a long time must be allowed for rehydration of the organisms. Recently Opfell, Hohmann, and Latham (1959) have shown that spores dried on to hard surfaces such as glass or plastic were much harder to kill than similar spores dried on to paper. When the spores were suspended in glycerine solution before drying they were easy to kill even on hard surfaces, suggesting that it is the humidity of the immediate environment that is important. These observations have been confirmed by others.

It can be seen from what has been already said that ethylene oxide is a powerful sterilizing agent, but that the number of variables to be considered makes its efficient use rather difficult. In practice a number of techniques have been developed and are in use in various circumstances. The simplest technique, applicable in the laboratory, is to use a desiccator or anaerobic jar as a chamber. The objects to be sterilized are placed in the jar, which is evacuated by a water pump. Ethylene oxide is admitted either as the pure gas or diluted with carbon dioxide or freon, until a manometer indicates that the required percentage has been added. After an exposure period at room temperature or in an incubator the jar is again evacuated to remove the gas, and air is admitted to restore atmospheric pressure before the jar is opened. The humidity of the chamber can be influenced, if not controlled, by placing in it a small dish of water. An alternative is to evacuate the jar and inject into it a measured volume of liquid ethylene oxide using a chilled syringe. This in practice is rather unsatisfactory because the liquid boils at 10°C. and makes accurate measurement very difficult. These methods using laboratory apparatus can be useful, but they cannot safely be used outside

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a laboratory or where trained staff are not available. For large scale sterilization two rather different processes have been devised on the continent of Europe and in the United States.

In Europe ethylene oxide has been used for many years to disinfest foodstuffs, disinfect hides, and, more recently, to sterilize medical supplies. A large metal chamber is used. After the load has been inserted the door is closed and a vacuum is drawn by an electric pump. A measured volume of liquid ethylene oxide is withdrawn from a cylinder and heated in a special vessel to vaporize it. The resulting gas is introduced into the chamber and raises the pressure towards, but not up to, that of the atmosphere. In some machines the pump re-circulates the air-gas mixture to ensure thorough penetration. After the exposure period the pump is used to vent the gas to atmosphere and to purge the system with one or two changes of filtered air. Because the chamber is always at subatmospheric pressure any leaks will be inward and the risk of explosion or intoxication will be minimized. The process is operated at room temperature and the exposure time is relatively long —usually overnight. In the United States the risks of using pure ethylene oxide are considered to be unacceptable and non-inflammable mixtures are used. In order to compensate for the lower concentration of gas the process is run at a raised pressure, after preliminary evacuation. The exposure time is further shortened by raising the temperature to 60°C. (140°F.). Humidity is controlled by injecting known amounts of water or steam.

Both these types of installation are complex and expensive. Schley, Hoffman, and Phillips (1960) have shown that plastic bags or oil drums can be used to improvise a sterilizing chamber, and Freeman and Barwell (1960) have converted an old steam sterilizer. Bracken, Wilton-Davies, and Weale (1960) have described the use of a plastic bag for sterilizing a heart-lung machine. However, because the process needs careful adjustment of several variables there is a tendency to fit sterilizing apparatus with automatic control gear so that reproducible sterilizing cycles are obtained.

Experience has shown that some of the early claims made for ethylene oxide as the perfect sterilizing agent were not justified. Attempts have been made to define a safe process in terms of concentration, time, temperature, and humidity, on the analogy of accepted standards for steam sterilization, but none can be accepted with complete confidence, and none will be given here. Thus Walter and Kundsin (1959) drew attention to the deficiencies of a small gas sterilizer and showed that when operated in accordance with the maker's instructions it did not sterilize test objects.

It is probable that each process must be tested thoroughly with suitable spore preparations in all parts of the load before the process can be regarded as acceptable, and that such tests must be repeated at frequent intervals. Even here a difficulty arises in deciding what is a suitable test object. It is generally agreed that spores of '*B. globigii*' should be used, but how many should be used, and how should they be prepared? As mentioned earlier, quite different results will be obtained if the spores are dried on paper as compared with plastic, and if 10^6 spores are used instead of 10^3. All that can usefully be said is that the presentation of the spores should resemble the load to be sterilized: plastic for disposable syringes, cloth for dressings, and so on. The numbers of spores used should be related to the probable level of contamination, though this is hard to assess: 10^6 spores per test object are commonly used, though it is probable that numbers as low as 10^3 are adequate.

Most attention has been given to the use of gaseous ethylene oxide for sterilizing medical and surgical supplies. The liquid substance has been used at low temperatures to sterilize liquids such as culture medium and also biological materials such as arterial grafts (Hufnagel *et al.*, 1953) and amniotic membrane (Freeman and Barwell, 1960). Much less experience is available of this application and it remains essentially one for laboratory use and laboratory control.

It is as yet rather early to say precisely what the place of ethylene oxide is in the sterilizing of materials for hospital use. On the commercial scale it is already being used for disposable items such as syringes and catheters, and where adequate bacteriological control is available this seems to be a satisfactory and safe process, although it is possible that the use of ionizing radiation may become economically more attractive, as well as more certain in its effects.

There will, however, always be items that need to be sterilized locally at hospital level rather than be bought from commercial sources already sterilized. If the bulk is small the sterilizing can probably be accomplished in the hospital laboratory using improvised equipment. If the bulk is greater, either from larger numbers of small items or single large objects such as heart-lung machines, it is probable that special sterilizers will be needed with automatic controls and monitoring of temperature, pressure, and humidity. These machines are expensive to buy (£2,000 to £3,000) and to operate (about 4s. per cubic foot of chamber space per run). Because of this and because of the constant need for laboratory control, it is to be hoped that such sterilizers will be centralized in groups or regions where they can be used both economically and safely. They are not

suitable for widespread use in small hospitals or isolated units.

The interested reader is referred to the papers of Phillips and Kaye already mentioned, to the review by Phillips (1957), and to a recent review by Thomas (1960) which contains a very full and up to date bibliography.

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Sterilization by gas ovens

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SYNOPSIS A forced convection gas-fired oven has been designed which will sterilize a very heavy load of syringes and instruments and containers. It is superior to a gas-fired, infrared, radiant-heated oven which was designed for sterilizing syringes.

Developments in the techniques of hot-air sterilization have been mainly directed to improvements in the conventional hot-air ovens themselves in order to ensure an even distribution of heat in the oven (Darmady and Brock, 1954). The incorporation of a fan has to a large extent overcome the uneven distribution of heat leaving pockets of cooler air which had led to failures in sterilization. Later, the introduction of the tunnel type of heater, using infra-red panel heating and a conveyor belt, speeded up the whole process, ensured a quick warming up of the materials to be sterilized, and, using a standard load, gave very reliable results (Darmady, Hughes, and Tuke, 1957). The heavy electrical load and the need for voltage stabilizers are disadvantages in many cases, and itwas thought that town gas as an alternative source of heating, and, to save space, a vertical form of oven, should be tried.

This paper therefore deals with two experimental installations: the first comprises a radiant heating unit with a vertically moving conveyor, and the second a forced convection unit with a horizontal conveyor. It should be pointed out that these are only two of the possible combinations of heating methods and conveyor disposition possible, since there is no reason why gas-fired radiant heating cannot be applied to a horizontal conveyor or forced convection to the vertical unit. Therefore the discussion of the results which follows later in the paper deals with these two aspects separately.

RADIANT HEATING UNIT

This unit is based upon a proprietary radiant heating panel, of which seven are used, each with a radiant surface 12 in. × 24 in. The syringes are carried vertically upward in front of four of the panels and pass downwards over the other three, being carried singly in special baskets on a chain-link conveyor. The syringes, assembled in the standard pattern Nuffield aluminium containers, are placed on an inclined plane-feed hopper from which they are automatically taken up into one of the basket carriers on the conveyor and are eventually discharged, after sterilizing, on the other side of the machine.

The overall dimensions are 2 ft. 4 in. × 2 ft. 9 in. × 7 ft. 6 in. high (Fig. 1). In order to use the full width of the proprietary radiant panel a double conveyor was installed, which will carry 150 syringes an hour. The unit is thermostatically controlled and for the experimental period has been fitted with a variable speed drive. Eventually it was decided that the best results were achieved with a conveyor speed which completed the heating cycle of a given syringe in 30 minutes.

FORCED CONVECTION UNIT

This unit was built around an existing conveyor 2 ft. 3 in. wide and comprises an oven space extending 8 in. above the conveyor some 8 ft. long (Fig. 2). The oven is constructed from Marinite insulating board supported by an angle iron frame, and the sides are extended 15 in. above the working space to incorporate the feed and return air ducts and the air heater and fan, thus giving a compact unit. The air heater has a No. 200 Hypact burner and is provided with a thermoelectric flame failure device and a fan failure switch, and is subject to thermostatic control. A ½ h.p. single-phase motor drives a centrifugal fan which provides forced recirculation of the hot gases through the working space in concurrent flow with the conveyor belt. A variable-speed motor drives the conveyor and can give heating times from a rapid five minutes up to 130 minutes.

As in the case of the radiant heating unit, this oven is also a prototype and was constructed to prove the operating characteristics. The horizontal conveyor has the advantage that objects of any size or shape can be placed upon it, and the oven height has been

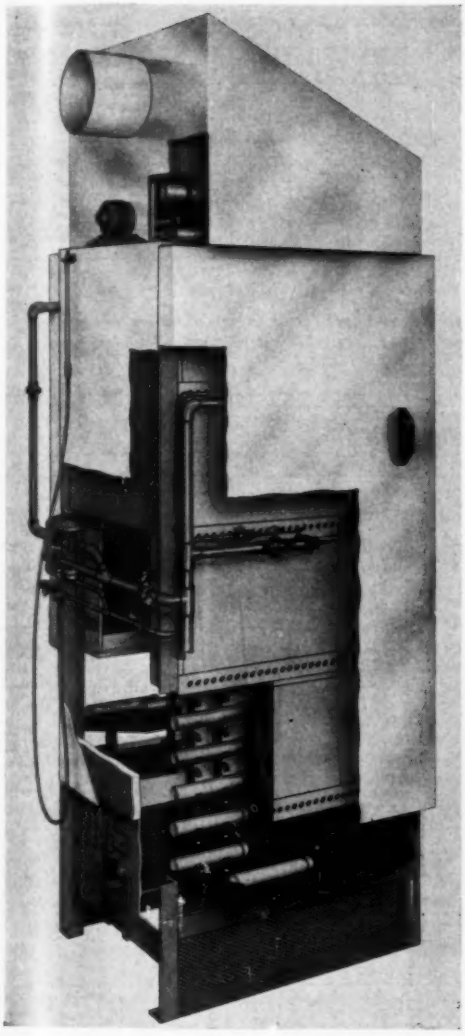

FIG. 1. *Vertical radiant sterilizer for hypodermic syringes.*

chosen to permit sterilizing of all the usual variety of containers, not only syringes but instruments, bowls, etc. It would be a simple matter to arrange for all sterilized objects to be automatically conveyed away.

TEST PROCEDURES

Copper-constantan thermocouples were buried in the plungers of syringes which were then assembled in the ordinary way. All sizes of syringes were tested in different ways but special attention was paid to the 2 ml. and the 20 ml. syringes as being the most widely different in size of those in general use. The experiments of Darmady *et al.* (1957) on the effect of new and old containers, blackening of containers, etc., were repeated and in the main confirmed with the radiant heating panels. Bacteriological testing was done using samples of earth, spores of *B. subtilis*, and spores of *B. stereothermophilus*. The spores were dried on blotting paper discs which were inserted into the base of the barrel of the syringe. The syringes were then fitted with a plunger and packed in the usual way. All tests were carried out at the beginning, the middle, and the end of a sterilizing run.

RESULTS

Figs. 3-7 are time temperature curves obtained with the thermocouples as described. Figs. 3 and 4 refer to the radiant heated oven, and Figs. 5, 6, and 7 to the convection oven. The first two graphs show that the

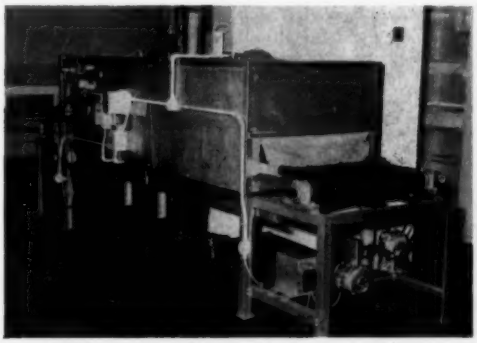

FIG. 2. *Forced convection unit.*

syringes receive radiation from the first panel within a few minutes and reach the required temperature of 160°C. or 180°C. within 10 minutes. The speed of the conveyor can be adjusted to allow for a slower warming up period but a much longer time at sterilizing temperature. The aim has been, however, to achieve a cycle of about half-an-hour, with a period of 15 minutes at 180°C. It will be seen from Fig. 4 that although this is achieved with old containers, new highly polished aluminium containers prevented the quick absorption of heat, resulting in a very short period at sterilizing temperatures. Fig. 3 shows that although the rate of heat uptake is somewhat different in different sizes of syringes the time of sterilizing temperatures is adequate without alteration of the controls.

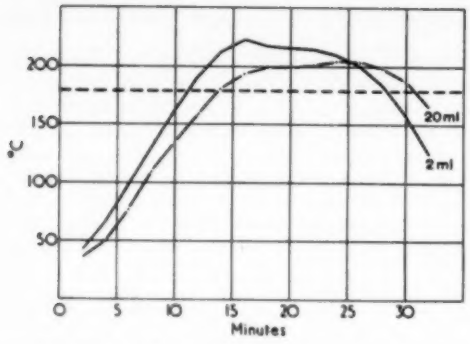

FIG. 3. *Results of tests using the radiant heating oven for syringes of different sizes maintained at a constant oven temperature.*

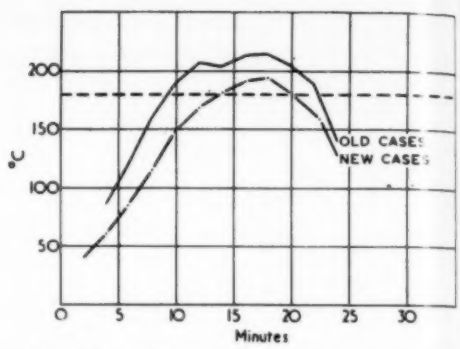

FIG. 4. *Results of tests using the radiant heating oven for syringes of identical size in old and new cases.*

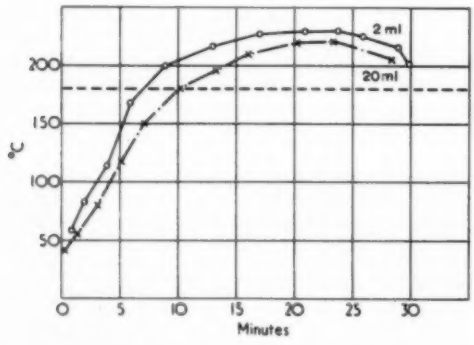

FIG. 5. *Results of tests using the convection oven for syringes of different sizes maintained at a constant temperature.*

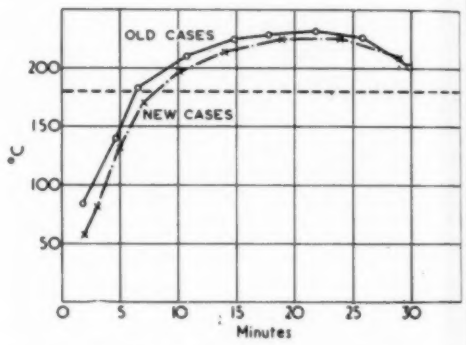

FIG. 6. *Results of tests using the convection oven for syringes of identical size in old and new cases.*

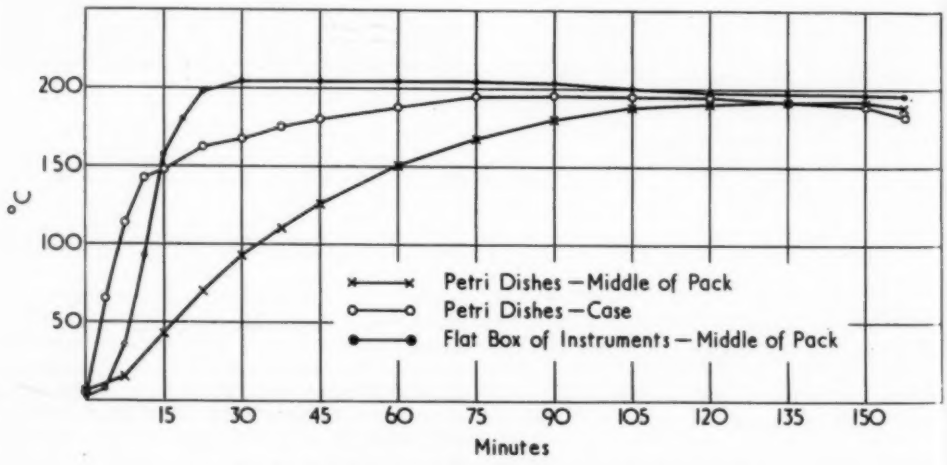

FIG. 7. *Results of tests using the convection oven for other articles.*

TABLE

A. *Comparison of Heating Systems*	*Radiant Heating Oven*	*Convection Oven*
Maximum throughput	150 syringes per hour	500 syringes per hour
Ignition	At seven points	At one point
Warming-up time of oven	80 minutes	20 minutes
Heating-up time of syringes	12-14 minutes	4-11 minutes
Effect of differing sizes of syringe	This can be seen by comparing Figs. 3 and 5. In both cases the small syringe has a peak temperature some 20°C. higher than the large syringe. The convection oven shows a greater retardation of heating-up for large syringes	
Effect of surface condition of goods	Fig. 4 shows that new tubes, being highly polished, are poor absorbers and this affects both heating time and final temperatures	Fig. 6 shows that, with convection heating, there is little difference between old and new tubes
Control of maximum temperature	Each heating panel has to be individually set to give the required heating cycle	Maximum hot air temperature cannot be exceeded. Temperatures steady
Control of heat input	Control of heating source is indirect, depending on heating by radiation of thermostat in a central, representative position	Control of heating medium direct by thermostat in hot air feed to oven
Fuel consumption per hour at maximum loading	1·15 therms	0·64 therms
Fuel consumption per 1,000 syringes	7·7 therms	1·3 therms
B. *Comparison of Conveyor Systems*		
Type of load	Purpose-built conveyor will take only syringes	Any object less than 6 in. high can be sterilized, *e.g.*, instruments, bowls, etc. Adjustment of conveyor speed only is required
Floor space	Small, at expense of added height	Relatively large, but height not great

Figs. 5 and 6 show the same tests carried out on the forced convection heater, and here it can be seen that there was essentially no difference in the results whether large or small syringes were tested or whether new or old containers were used. Fig. 7 relates to larger objects with thermocouples at the positions indicated.

Bacteriologically the results were eminently satisfactory in both types of oven. No growth was ever obtained with a syringe which had been exposed to at least 180°C. for 10 minutes. It is important to check control spore papers, as there is occasionally deterioration and a false sense of security may be felt when spores are too readily killed on heating.

DISCUSSION

The advantages of the forced convection oven over the infrared radiant heating oven are clearly shown in the table of comparisons. The most important seem to be that the temperature of the second half of the convection oven is very constant, and that objects of varying shapes and sizes reach and maintain this temperature. Because the temperature is maintained by a forced circulation, and due to a sensitive temperature control, there is very little difference in the eventual temperature and time of exposure between new and old containers when they are mixed in one load, or between syringes and containers of very different sizes. This makes for greater ease and flexibility in working. The radiant-heated vertical oven, although it gave very good results, was very much more limited and could only be used for syringes, and preferably syringes of similar size and the same type of container. The running cost of the forced convector oven per syringe was approximately one-sixth that of the infrared panel heater.

Our thanks are due to the Nuffield Foundation and the South West Metropolitan Regional Board, which gave grants to cover some of the expenses, and to the North Thames Gas Board. We should also like to thank Mr. Whiter and Mr. Addiscot, of the Engineering Department of St. Mary Abbot's Hospital, for their help.

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Disinfection of bedpans

E. M. DARMADY, K. E. A. HUGHES, J. D. JONES, D. PRINCE, AND PATRICIA VERDON

From the Portsmouth and Isle of Wight Area Pathological Service

SYNOPSIS A standard dish-washing machine fitted with an automatic cycle has been used to clean and disinfect bedpans. Visual and bacteriological examinations have shown that the machine produces superior and more reliable results than in trials of bedpans submitted to previously described methods.

The bedpan is probably one of the most commonly used articles in ward practice, although little attention has been paid to its cleaning and disinfection. This is partly due to the failure of the medical and administrative staff to penetrate through the portals of the sluice room, and partly to the natural reticence of junior nurses to disclose the sordid nature of their duties. Many of the recommendations made in the past for the cleaning and treatment of bedpans have been devised in the academic circle of committees who are out of touch with the practical aspects and difficulties of nursing procedures. A survey made by the Nuffield Provincial Hospitals Trust (1958) and by Darmady, Hughes, Jones, and Verdon (1959) revealed that many of the pieces of apparatus provided were inadequate or the methods used were so cumbersome that nurses were unable to fulfil the discipline demanded of them.

When this survey began five methods were available.

(1) Washing the bedpan by hand with a mop kept for the purpose and usually stored in one of a series of quite inadequate disinfectants followed by rinsing by hand under running water. As no effort was made to disinfect the pan, it was considered unnecessary to test it for the presence of faecal organisms.

(2) Sluicing the bedpan in a specially constructed piece of apparatus attached to the wall, in which it was sprayed with either cold or hot water and operated by the nurse who lifted one or two levers. No time cycles were recommended and the period of flushing depended on the personal preference of the operator. It was not surprising that a large number of pans showed visible faecal remains and that of the 93 tested all grew faecal organisms.

(3) The bedpan was sluiced by the previous method, but after treatment it was transferred to a boiler usually constructed to hold six or more pans. As the pans were seldom cleaned and as the mass of metal was considerable, the boilers took time to return to boiling water temperature (sometimes over one hour). Furthermore, as the nurse frequently added or removed bedpans during the heating-up of the boiler, it was not surprising that, in the trials carried out, of the 18 treated by this procedure 11 grew faecal organisms.

(4) The bedpan was sluiced as in method 2, but the apparatus was also fitted with a nozzle for injecting live steam into the chamber holding the pan. As, however, the operation and timing of the various procedures were left entirely under the control of a junior nurse the time cycle was reduced to a minimum, and, for subsequent thermocouple studies, the temperature reached by the pan was never adequate to kill faecal organisms. This was confirmed by the fact that out of 48 samples examined faecal organisms were recovered in all cases.

(5) As a number of manufacturers appreciated the inadequacies of personal operation, sluices were devised and fitted with automatic controls and locking doors so that the cycle could not be interrupted. Three different types were tested, their cycles being regulated so that a jet of water either cold or hot and under pressure played over the surface of the pan. The volume of water was sometimes considerable, but in spite of it faecal remains were frequently observed. This procedure was followed by an injection of steam at atmospheric pressure. The cycles varied from 20 seconds to 35 seconds. One of the major disadvantages of this system was that if faeces were still adherent to the pan, the steam caused the protein to coagulate and baked the residue firmly on to it, making removal well nigh impossible. It was also interesting to note that when such remains were cultivated faecal organisms were isolated. Cultures of the pans showed that the number of occasions on which faecal organisms could be isolated was reduced when compared with the previous methods described, and varied from approximately 25% to 51% (Darmady *et al.*, 1959).

These findings were somewhat surprising, and trials therefore were carried out to ascertain what

temperatures were actually recorded in the pans. In each case the ball of the thermocouple was attached to the rim of the pan by a paper clip. On no occasion in any of the machines using automatic cycles did the temperature reach 80°C. Further experiments using a machine in which the control of steam could be manually operated showed that at least 35 to 45 seconds were required to reach this temperature, and this can be accounted for by the size of the chamber used to enclose the bedpan and to the mass of metal making up the bedpan itself.

Finally, two further practical disadvantages of this type of machine must be mentioned. First, it is the practice for the nursing staff to carry out a 'bedpan round'. The nurse delivers to the various patients a number of pans, and on completion she returns with up to three or four pans for disinfection and subsequent re-use. As they have to be disinfected in these machines singly she is intolerant of the delay caused by the automatic cycle (45-50 seconds), since she may be occupied for four or five minutes awaiting the completion of her load. On occasion this has led to pans used for micturition not being included in the disinfection cycle at all. Secondly, as these machines are covered by specifications laid down by the British Standards Institution the outflow of the soil pipe has an internal diameter of 3 in., and if the nurse has not removed excessive toilet paper and/or sanitary towels, it is frequently blocked. This has necessitated instructions being issued that such objects should be removed by hand before the pans are placed in the machine—a practice which leaves much to be desired. It has been stated that the provision of the restricted outlet was partly due to hospital engineers who felt that it would prevent the blockage of soil pipes at some inaccessible place, and in part that the restriction would lead to a better emptying of the sump. In future an outlet must be provided in such machines which will completely dispose of solids and any sanitary towels.

At about this time, it was noted that the current methods of disinfecting crockery in the tuberculosis wards was hopelessly inadequate (the method employed was similar to that described in 3 above), and that it was possible to culture *M. tuberculosis* from plates artificially infected with tuberculous sputum. In view of the obvious risk to the operator, it was decided to make use of a washing machine fitted with a heater tank. The cycle of the machine was operated by hand, and although this was found to be satisfactory when controlled by a competent person, some operators were unreliable. It was essential, therefore, to make the cycle automatic. The cycle found to be satisfactory was as follows:

(1) The heater tank was set at 190°F. (88°C.).

(2) As soon as the machine was loaded, and the lid closed, approximately one gallon of water at 190°F. was allowed to enter, and was recirculated by means of a rotating propeller. The mass of crockery cooled the water to below 120°F. (49°C.); the optimum temperature for washing varied according to the load used. The chamber was also fitted with a heating coil which heated the recirculated water. As protein coagulates at approximately 140°F. (60°C.) it was important that the first, or washing cycle, should not be continued for too long. Investigation with thermocouples showed that the optimum time was between 2 minutes and 2 minutes 30 seconds. In practice the first cycle was set for 2 minutes 15 seconds.

(3) At the end of the washing cycle a valve opened and allowed the water and food debris to run to waste. These were replaced by a further gallon of water at 190°F. (88°C.) and allowed to recirculate. As the contents were now clean and at temperatures between 120°F. and 140°F. the heat of the water could be raised to ensure disinfection. Experiments showed that this cycle should last six to seven minutes, during the last four of which the temperature of the articles was found to be between 178°F. and 184°F. (81°-84°C.). At the end of the disinfecting cycle the water was allowed to drain away, and since the temperature was high and circulation of air was maintained by means of the rotating propeller the articles were 'flash dried'.

Bacteriological tests using 49 plates showed that when plates were artificially contaminated with *Strep. faecalis* and put through the machine the organisms were consistently killed.

It occurred to us that such an automatic cycle could be adapted for bedpans. A parallel probably existed in the milk industry which had found that, provided bottles were clean, the relatively low temperature of 180°F. was adequate to kill pathogenic organisms, including *M. tuberculosis*, but excluding some spore bearers, and to test this hypothesis a basket was constructed to hold four standard bedpans (B.S.I.). Investigations showed that the same automatic time cycle was satisfactory, and since the load was standard in size the temperatures attained were far more reproducible than with crockery.

Accordingly the following procedure was introduced. As soon as the bedpan round had been completed, the solids were discharged into an open sluice or preferably into a sluicing machine fitted with a simple lavatory flush. This takes approximately eight seconds for each bedpan. The bedpan is then transferred to the machine. When the four pans are loaded, the automatic cycle is switched on. Since it is automatic and takes just over nine minutes, the nurse is available for other duties. At the present time 12 such machines have been installed in the Portsmouth Group of Hospitals (three for over two years), and

two each in the remaining areas of the Hospital Management Committee of the Wessex Region.

Visual investigation showed that all were free from faecal material, and of 184 tested only 11 grew faecal organisms. On each occasion the bacteriological tests were performed as follows:

Sterile 'stick' swabs made with non-absorbent cotton wool were dipped into bijou bottles of sterile nutrient broth. The swabs were then smeared all over the floor and under the rims of the bedpans. On completion the sticks were broken off into the nutrient broth. They were then incubated for seven days and subcultured on to MacConkey agar plates.

Because the object tested was large, and indeed might be contaminated with faecal organisms by an aerosol after heat treatment, particularly as the tests were carried out in the sluice room, these results appear satisfactory. But, it should be pointed out that with this technique it is not possible to determine the total bacterial count as a single colony may still provide a positive result.

DISCUSSION

The provision of a central sterile supply of bedpans has not so far been considered practical, principally because of the distribution involved, nor does the use of disposable bedpans appear attractive, largely because of cost. Chemical disinfection of bedpans is also unsatisfactory, since it is necessary to emulsify the faeces thoroughly before treatment. Again this is not practical, and with disinfectants such as lysol can be dangerous if the pan is not thoroughly rinsed before re-use. An advantage of the adapted automatic crockery machine is that the apparatus does not require a steam supply, and in the future design of hospitals this may be a great saving in capital cost. At the present time, only about one-third of hospitals in the Wessex Region are equipped with steam to the sluice rooms. Indeed one of the major hospitals in the Group (510 beds) is deficient in this respect. Further, as the machine is an adaptation of a standard manufactured article, the cost is relatively lower than the completely automatic wall type of bedpan sterilizer fitted with steam. Indeed the use of hot water only reduced the risk of faecal remains being baked onto the pan, and the fact that the adapted machine handles four at once has found favour with overworked junior nurses. It is important that the bowl should be filled by water level rather than by time cycle since any variation in water pressure may cause the washing and disinfection treatment to be invalidated.

Some may prefer to add a second washing cycle before the final disinfecting stage. With such machines this should not present mechanical difficulties, In most of these investigations no detergents or wetting agents were used, because, first, it was essential to ensure that disinfection was wholly a result of the heat treatment, and secondly, it was not thought that they would be successful since the majority depend upon the fat-solvent or liquidizing action to mobilize the contaminating material (this is particularly true of detergents designed for washing crockery). Faecal material, because of its high protein and low fat content, is particularly difficult to remove. Another difficulty encountered has been the cleaning of pans after faecal material has been allowed to stay in them for some hours or they are contaminated with blood. Trials were made by lining the pan with paper or tissue. However, prolonged contact resulted in the faeces soaking through such materials and becoming adherent to the pan. The only satisfactory method of retaining faeces for visual inspection and yet allowing the bedpan to be easily sterilized, has been to ask the patient to void urine first and then to line the pan with polythene sheeting. The faecal material can then be removed from the pan by lifting the four corners of the sheeting, and transferring it to a large wax carton, which can subsequently be burnt.

The major disadvantage of the adapted crockery washing machine is that two processes are involved, with the possible danger of cross-infection during the transference of the bedpan from one piece of apparatus to another. In spite of this, in our hands it has consistently produced cleaner and more hygienic pans than any previously encountered. It is clear that in the future the manufacturers must concentrate on a machine which will allow the major solids to be removed and the pan treated with recirculated hot water, and will treat four bedpans at the same time in a space no greater than that occupied by a crockery washing machine.

The present design of the bedpan makes it difficult to clean. The overhanging internally rotated seat makes it difficult for water even under pressure to penetrate to this area, but a pan of which the seat is externally rotated would be easier to clean, although it is appreciated that such a model might not be so easy to handle and the contents might spill while it is being carried.

We are grateful to the Wessex Regional Hospital Board Research Committee for an expenses grant, and providing the thermocouples; and to G. R. Scruby Esq., Director of Messrs. Dishlex Limited, for the loan of apparatus, and the construction of the special baskets.

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The organization of central sterile supply departments

J. D. WELCH

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The last few years have witnessed an increasing interest in organizing sterilization. Every new hospital now being planned in the British Isles includes provision for a central sterile supply department and many existing hospitals are also planning such departments. Several of these are likely to be opened within the next year or two. At the present rates of progress there will be few hospitals without central sterile supply departments by 1970. All this cannot be done without a great deal of work. Invariably it has been the hospital pathologist who has taken the initiative in the planning, organizing, and running of these departments. It is appropriate therefore that space should be devoted to this subject in the *Journal of Clinical Pathology*. Several articles have recently appeared dealing with the organization of central sterile supply departments. An excellent article by V. D. Allison appears in the *British Medical Journal* of 10 September 1960, and gives an account of the pioneer work which has been done at Musgrave Park, Belfast. It is hoped that the article that follows will be complementary to that of Dr. Allison. For those more closely concerned with the design of central sterile supply departments reference may be made to the article appearing in the *Architectural Journal* of 7 July 1960.

AMERICAN PRACTICE

The original idea of concentrating the arrangements for sterilization in one department arose in the U.S.A., and, before considering British practice, it may be of value to take a quick glance at American methods. But this must be done against the background of American hospitals. These are in competition with each other for those patients who can afford to pay the high fees charged. American hospitals, therefore, are lavish and it is usual to provide the staff with anything that they may require for a particular procedure. But all this equipment is seldom used and appreciable waste results. Again, as the costs can be passed on to the patients, there is little incentive to achieve economy by organizing a proper work flow. In the U.S.A. it does not seem to be realized that a central sterile supply department should be run more like an efficient factory than like a hospital department. It is certain that under the conditions of a National Health Service central sterile supply departments must be able to practice and demonstrate more efficient methods than are common across the Atlantic.

MEANS OF STERILIZING

Before considering how central sterile supply departments should be run it may be of value to enumerate the means of effecting sterilization. So far as hospitals are concerned there are five possible methods: by chemical means, by irradiation, by using gases, by steam, by hot air.

CHEMICAL STERILIZATION This is far from sure; it takes a long time; it must be done wet. Although a number of new and improved chemicals have been produced, it will generally be agreed that since chemicals disinfect rather than sterilize they should be used only if no other methods are possible.

IRRADIATION Recent increases in the peaceful use of atomic energy have brought the possibilities of irradiation to the fore. Over more traditional methods irradiation has certain advantages. It is sure in operation. It does not require heat. But irradiation also has important disadvantages so far as hospital products are concerned. It turns glass brown and, in time, breaks up the fibres of textiles. But the chief disadvantage of irradiation is that to be economical in operation the product to be sterilized should be fairly standard and a vast initial capital outlay is necessary. Unfortunately hospital products for sterilization are far from standard. They are indeed of a highly variable nature. Many are of glass or are textiles. As all hospital authorities know too well, vast capital sums are hard to come by. It is concluded, therefore, that, although sterilization by irradiation has considerable advantages for large scale manufactured products, it is unlikely to be of much practical value in hospitals for some time to come.

GASEOUS STERILIZATION This was developed in Germany and the U.S.A. during the second world war, the gas which proved most promising being ethylene oxide. But here again difficulties and disadvantages are apparent. To be properly effective ethylene oxide requires to be of a high concentration, and humidity and temperature need to be carefully controlled. Ethylene oxide at high concentration is highly explosive, so under hospital conditions it is usual to use a mixture of 20% ethylene oxide and 80% carbon dioxide. In such concentrations this gas is proving unreliable, although with increased experience and more reliable control improved results may be obtained. So far as hospitals are concerned, therefore, this gas is likely to be of more value for the sterilization of things which cannot be sterilized any other way. Heart-lung machines are a case in point. Gas is unlikely to prove a substitute for steam under pressure or hot air.

STEAM AND HOT AIR Steam under pressure is the classical agent for sterilizing most of the needs of hospitals. But, there are advantages in using hot air for certain articles such as syringes and instruments. As the uses of steam and hot air are dealt with fully in other articles in this issue further reference to them is not made in detail here, except to state that they are likely to remain the principal sterilizing agents used in hospitals for a long time.

AIMS AND SCOPE OF CENTRAL STERILE SUPPLY DEPARTMENTS

It is at the outset necessary to be perfectly clear as to what central sterile supply departments are intended to achieve. If this is not done these departments may find themselves being called upon to attempt all kinds of tasks for which they were never intended. The aims of central sterile supply departments are: First, to conduct hospital sterilizing practice so that it can be properly controlled and supervised, thereby contributing to a reduction in the incidence of hospital cross-infection, and second to take a measure of work off the nursing staff thereby giving them more time to devote to their patients.

A central sterile supply department should, therefore, be charged with the production of all the sterile requirements of the hospital it serves, other than pharmaceuticals, bedpans, urinals, and theatre surgical instruments. Pharmaceuticals are the responsibility of the pharmacist; it is impracticable to centralize the cleaning of bedpans and urinals; and so many surgical instruments are used in the theatres that it would be laborious and costly continually to transport them about the hospital.

PACKAGING

It will be appreciated that, even if pharmaceuticals, bedpans, and theatre surgical instruments are excluded from the tasks of central sterile supply departments, the list of articles still requiring to be sterilized remains a very long one, ranging from such things as small spigots to large maternity packs. If such a variety of products are to be packed before they are sterilized (and it would be inadvisable to package them after sterilization) then packaging methods must be considered. The method of sterilization will often be dictated as much by the way in which the article to be sterilized is packed as by the nature of the article itself.

In the U.S.A. it is customary to employ a composite pack for each surgical procedure and to enclose them in two layers of linen. Fig. 1 shows an American composite pack. Such a pack contains everything that the surgeon or nurse can require for a procedure, and necessitates the production of an excessive variety of packs, many of the sterilized items of which go backwards and forwards without being used. A simpler system is required under National Health Service conditions. If soft (disposable) and hard (returnable) goods are packed separately, the nurse can select what she wants for each particular procedure and a more flexible and economical system results.

SOFT PACKS Experiments have shown that the soft goods should be packaged in paper or in paper bags and then be put into cardboard boxes. Two barriers are thus provided to protect the sterile contents from contamination. If one of the barriers is of as firm a substance as cardboard the package is also less likely to be punctured. Fig. 2 shows a soft pack being prepared.

A list of suitable soft packs is given in Appendix A. This list is for general ward use only and will, of course, require supplementing for particular specialties. All the packs mentioned in Appendix A, together with surgical gloves etc., can best be sterilized by steam in autoclaves. They will not stand high temperatures. (Full details about high-vacuum steam sterilization are given in the paper that deals specially with this subject on page 11.)

It may be helpful to include some remarks on the expected shelf life of soft goods sterilized and packaged in this way. The answer will, of course, depend as much on the conditions of storage as on the methods of packing. Provided that the conditions of storage are good, *i.e.*, such as are met with in a clean utility room of a well-run ward or a theatre store room, the sterilized packs could probably be stored for a month without danger of contami-

FIG. 1. *of syring*

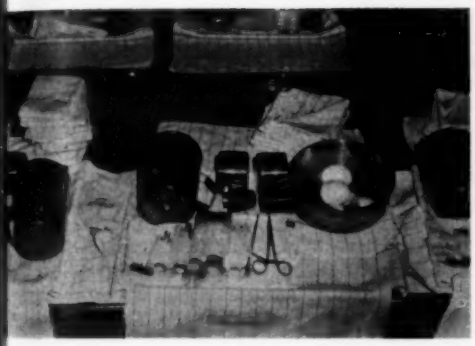

FIG. 1. *A composite American pack. The large numbers of syringes, bowls, gallipots, and towels should be noted.*

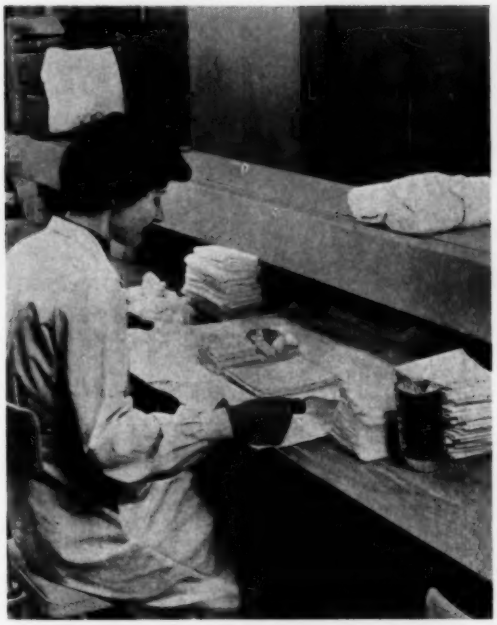

FIG. 2. *A British soft pack being prepared. Everything is disposable. Compare with Fig.* 1.

FIG. 3. *A simple instrument pack. The design of the instruments to open out should be noted.*

FIG. 4. *Dispensers for syringes and small packed items.*

tion. If a longer shelf life is required, as may be necessary for an emergency reserve stock, this can be met by using a third barrier of paper, either inside or outside the cardboard box. If three barriers are used in this way the packs can probably be stored in safety for as long as six months without re-sterilization. Shelf life can best be indicated to users by stamping each pack with an expiry date.

HARD PACKS If soft goods are packed in paper and cardboard, how should the hard goods, which are robust enough to stand sterilization by hot air, be packed? It has been found convenient to pack syringes and instruments in extruded aluminium tubes each sealed with an aluminium capsule. These aluminium tubes are widely used for the sterilization of syringes by hot air at 180°C. The instruments required for ward procedures can all be fitted into a 20 ml. tube, provided that scissors, stitch removers, and artery forceps are re-designed so that they open out like a pair of travelling scissors. Such re-designing can easily be done by any instrument manufacturer and should not increase the cost of the instruments. Fig. 3 is a photograph of an instrument pack.

A list of suitable instrument packs is given in Appendix B. This list is suggested for general ward use and will require supplementing in the case of casualty departments and certain specialities. Full details of sterilization of syringes and instruments by hot air are given on page 39.

As it is difficult to stamp syringe containers with expiry dates, a solution has been found by using dispensers which, by loading from the top and unloading from the bottom, ensure that syringes and instruments are used in proper rotation. This is adequate for practical purposes, since the turnover of such items is generally more rapid than in the case of soft packs. Fig. 4 is a photograph of a syringe dispenser and a dispenser for small packed items. If all the articles mentioned above are to be provided packed and sterile for use in all departments, it follows that there is no need for the wards also to be provided with their own means of sterilization. Indeed it would be a mistake to do so since sterilization there cannot be controlled or supervised, and inefficient processing is thereby encouraged.

ORGANIZATION OF CENTRAL STERILE SUPPLY DEPARTMENTS

Plenty of evidence is already available to show that cross-infection may be spread through the agency of nurses' fingers. Yet nurses are continually called upon to rinse equipment which is known to be contaminated. This is inevitable if sterilization is undertaken on the wards. But when central sterile supply departments are available it should no longer be necessary for nurses to rinse a used syringe or instrument. It is, therefore, suggested that, as soon as an instrument is used on a hospital ward, it should be disposed of as quickly as possible without the nursing staff undertaking any rinsing. This should reduce the risk of cross-infection where it matters most, *i.e.*, on the hospital wards. This will mean that there are three classes of equipment, the first being returned to central sterile supply departments will, at least, be potentially contaminated, *i.e.*, 'dirty'. All used articles should, therefore, be washed in a wash-room set aside for that purpose. No article should leave this room until it is clean and dry. It can then be moved in a clean and dry state to its own particular room for further processing: gloves to the glove room and instruments and syringes to the instrument and syringe room. The second class of equipment arriving in the department will be from manufacturers and laundries, *i.e.*, it will be 'clean'. These two classes of equipment, the one originally 'dirty' but now 'clean', and the manufactured disposables will

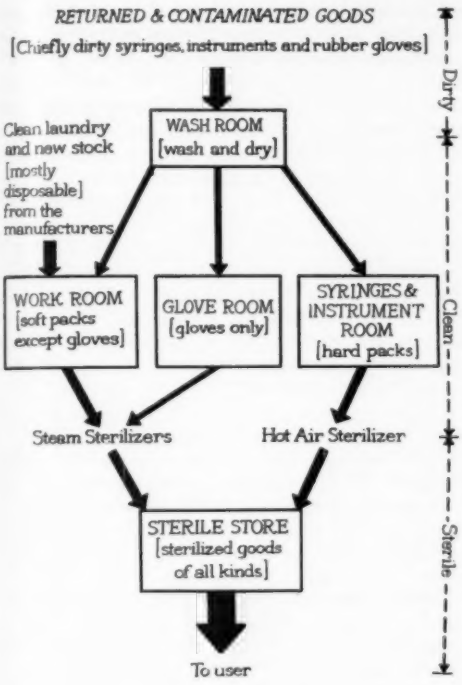
RETURNED & CONTAMINATED GOODS
[Chiefly dirty syringes, instruments and rubber gloves]
Dirty
Clean laundry and new stock [mostly disposable] from the manufacturers
WASH ROOM [wash and dry]
WORK ROOM [soft packs except gloves]
GLOVE ROOM [gloves only]
SYRINGES & INSTRUMENT ROOM [hard packs]
Clean
Steam Sterilizers
Hot Air Sterilizer
STERILE STORE [sterilized goods of all kinds]
Sterile
To user

FIG. 5. *Organization and work flow diagram.*

then be married together, packed, and sterilized. The third class consists of all the sterilized equipment. The central sterile supply department requires to be organized to keep these three classes of equipment separate yet organized into a continual and logical work flow. The processes are illustrated in Fig. 5.

Experience has shown that once the goods have been packed and are ready for sterilization, it may be advantageous to store them temporarily in a non-sterile store. Here they can be held until required for sterilization. With modern high-speed autoclaves, sterilization takes very little time and, if packs are sterilized shortly before issue, there is less danger of them becoming 'time expired' before they are used.

DISPOSABLES

It is only within the last few years that attention has been given to disposable equipment. But the possibilities are now appreciated by many manufacturers as well as by the hospital authorities who are beginning to realize the heavy cost of re-processing. During the course of experimental work various disposable kinds of equipment have been tried.

DISPOSABLE SYRINGES These are already reaching the British Isles from America. The 2 ml. size, already packed and sterilized, retails at about 6d. The price of other sizes as and when they become available will vary directly with the size of the syringe. Such considerations will exclude, on ground of cost, all the larger sizes. Hospitals can process their own syringes, including a due proportion of the larger sizes, for an average of about 4d. or 5d. It follows that disposable syringes are unlikely to be an economic proposition under National Health Service conditions.

DISPOSABLE NEEDLES Nearly half the cost of the syringe processing mentioned above is attributable to processing the needle, which costs the hospital nearly 2d. each time it is processed. Disposable sterile needles are not yet on the market in England. But when they become available they are not likely to cost much more than 2d. In effect, therefore, the disposable sterile needle is a better economic proposition than the disposable syringe and its future in the hospital service looks promising.

PAPER In the past linen has been used extensively in hospitals for such purposes as drapes and hand towels. But this linen has to be laundered and then issued for use again. Laundering alone may cost as much as 2d. per article. Much of this cost and a lot of the labour involved in recirculating the linen would be saved if paper were to be used instead of linen. Manufacturers are aware of this and paper of greatly improved types is now being made available for hospital use. Fig. 2 shows a dressing pack of which most is paper. It would be a mistake to attempt to give details of the types of paper available for different purposes, since progress is so fast that any recommendations made would probably be out of date when published. Suffice it at this stage to say that paper is already available and probably better and cheaper than textiles for the following purposes: Instead of cotton wool balls for skin cleaning, instead of hand towels (sterile and unsterile), instead of surgical dressing towels, and as a packaging agent to prevent bacterial contamination. Those charged with the conduct of central sterile supply departments should keep in touch with developments in papers and see which are the most suitable for their purposes.

ALUMINIUM FOIL This is already widely used as a disposable substitute for the smaller sizes of stainless steel containers as also can be seen from Fig. 2.

CARDBOARD BOXES These are now widely used as semi-disposable substitutes for hospital drums. They are lighter in weight, cheaper, and more effective in preventing bacterial contamination. They require, however, to be used only in conditions of dry steam. If hospital steam is wet—and this is not unusual—it will soon be demonstrated by the cardboard breaking up. In the experimental department at Addenbrooke's Hospital, Cambridge, no cardboard box has yet had to be replaced although many have been in use continuously for over six months.

PLASTICS Plastics have many uses either in a clean or sterile state. They are so cheap that they can be used once and thrown away. Used clean they are available as mattress and pillow covers to protect their contents from bacterial contamination; draw mackintoshes; gloves for such purposes as rectal examinations; and disposal bags for dirty dressings and household refuse. Sterilized by gamma-irradiation they are available as catheters and urological drainage bags.

COLLECTION AND DELIVERY

It is stated earlier that one of the aims of central sterilizing is to take work off the nursing staff. If the nursing staff were to undertake their own delivery and collection, the aims of the system would not fully be achieved. All central sterile supply departments should, therefore, undertake a collection and delivery service. Distribution systems are still the subject of experiment. They can be summarized under three heads: The 'one-for-one exchange' system, the 'milk-round' system, and the 'grocery' system.

'ONE-FOR-ONE EXCHANGE' SYSTEM Broadly this system consists of giving each ward a standard pannier containing their sterile supplies for 24 hours. Each 24 hours the pannier is exchanged for a full one and the unused supplies are taken back. The system has advantages. The wards cannot over-stock; packs cannot remain out long enough to become potentially contaminated, and exchange at the wards is speedily carried out. Yet the system also has considerable disadvantages. It cannot be used for all ward supplies, since these are too numerous. The very small and the very large items have to be delivered by a system of 'topping-up'. Exchange is therefore not so rapid as it might be. The panniers cannot themselves be sterilized and it seems doubtful whether they afford adequate protection to their sterile contents under adverse conditions. More work is also involved than is necessary since some of the contents of the pannier go backwards and forwards without being used. On balance it is not considered that the pannier system of delivery will prove suitable for widespread use.

'MILK-ROUND' SYSTEM This system, as its name implies, envisages delivery being undertaken by a system similar to that of milkmen, the delivery trolley being loaded with a standard load which is used to 'top-up' each ward on arrival. But experiments with this system have been disappointing. The requirements of most hospital wards are so varied that it requires a huge trolley to accommodate them all. This trolley, even with modifications, has proved too big to be manipulated satisfactorily in narrow hospital corridors. Work on this system of delivery has accordingly been abandoned and a solution sought by a third system.

'GROCERY' SYSTEM As its name implies, this is a system similar to that whereby a housewife receives her groceries. It is not without its drawbacks. In the first place it requires the ward to order supplies in advance. This involves the nurses in additional work which it would have been better to avoid. Secondly there is no way of preventing a ward from over-ordering; it is only possible to check it later. But the system has the advantage that ward orders can be made up in the orderly atmosphere of a central sterile supply department. Of the three methods the 'grocery' system involves spending least time delivering goods in ward passages. It is hoped, in time, to educate the nursing staff not to hoard unnecessary equipment.

STAFF

The importance of the staff of central sterile supply departments cannot be overemphasized. When the Nuffield Provincial Hospitals Trust published their booklet 'Present Sterilizing Practice in Six Hospitals' it was stated on page 52 'As always, the human element decides the quality of the service given.' This will always be the case so far as sterilization procedures are concerned. Of the staff the most important person is the supervisor. It is premature to attempt to suggest what technical qualifications the supervisor should hold. Some of those now doing such work are one-time theatre sisters, some are male nurses, some are laboratory technicians. The post calls for just those qualities of character and intellect which are always in demand. These are more important than technical qualifications. For the ordinary work of the department women orderlies are suitable. There are no longer heavy autoclave doors to be opened and shut and the work is usually done more conscientiously by women. When departments

are fully operative and if the work is properly organized and mechanized, then a scale of labour as low as 1·5 orderlies per 100 beds to be served may be possible. This kind of a figure is, however, unlikely to be reached in the early stages of establishing a central sterile supply department.

COST

It is appreciated that persons planning central sterile supply departments want to know prime and maintenance costs. These departments have not yet been running long enough for reliable figures to be forthcoming.

I should like to thank the many people who have assisted and are still assisting in all the work that is being done at Cambridge. The staff at Addenbrooke's Hospital must be mentioned in particular. They have been most patient and conscientious in the various experimental work and have always been happy to try anything once. Considerable help has also been forthcoming from the development staff of the many manufacturers who have submitted their different products for trial.

Appendix A: Contents of Four Dressing Packs

1 SMALL PACK

Use: For minor dressings and similar procedures
Contents: 1 dressing towel—20 in. × 20 in.
1 aluminium foil gallipot
2 gauze swabs—4 in. × 4 in. × 8-ply
2 wool balls.

2 MEDIUM PACK

Use: For such procedures as lumbar punctures, aspiration, catheterization, etc.
Contents: 2 dressing towels—20 in. × 20 in.
2 aluminium foil gallipots
6 gauze swabs 4 in. × 4 in. × 16-ply
6 wool balls.

3 LARGE PACK

Use: For major dressings and similar procedures
Contents: 2 dressing towels—20 in. × 20 in.
1 gallipot, aluminium foil
4 gauze swabs—4 in. × 4 in. × 16-ply
6 wool balls
2 wool squares—8 in. × 12 in.

4 COTTONWOOL BALLS

Use: For supplementing any of the three standard dressing packs
Contents: 6 cottonwool balls

Appendix B: Contents of Four Instrument Packs

1 4 pairs of dissecting forceps

2 4 pairs of dissecting forceps
1 pair stitch scissors
1 pair clip removers

3 1 pair artery forceps
1 pair sinus forceps
1 probe

4 Intravenous cutting down set

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Preserving the sterility of surgical dressings wrapped in paper and other materials

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SYNOPSIS Materials for wrapping surgical dressings were studied by a method which measured the recontamination rate in packs stored for periods of up to several weeks. Of the materials tested a thick crêpe paper was better than calico and balloon cloth fabrics. Dressings wrapped in paper were not significantly contaminated after storage in dusty places for periods of up to three weeks. For added protection during handling and for convenience in transport and storage, paper packs may be sterilized and kept in paper-lined cartons. The shelf life in good storage conditions would be many weeks at least.

When gloves, dressings, and other fabrics have been sterilized in an autoclave, their sterility must be maintained during subsequent transport and storage. To achieve this the sterilized goods must be enclosed in containers or wrapped in materials which are as nearly germ-proof as possible, consistent with penetrability by steam, convenience in handling, and reasonable economy. In this paper we report the results of bacteriological experiments designed to evaluate various wrapping materials and containers, and we make some observations on their use.

METHODS AND MATERIALS

Test swabs were used to detect bacteria which succeeded in penetrating the container or wrapper after sterilization. The swabs were the cut-off ends of throat swabs made with wooden sticks. Several test swabs were enclosed inside each package, and were always in contact with the wrapper or the wall of the container. After sterilizing in a high pre-vacuum autoclave the packs and the containers were allowed to stand unopened for lengths of time varying from one day to three months in surgical wards, corridors, and other parts of the hospital. The places chosen were draughty and dusty. There were abundant bacteria in the atmosphere as shown by colony counts on 3½in. nutrient agar settle plates incubated aerobically for three days at 37°C., which gave results between 40 and 100 colonies per hour.

The exposed packages were sampled in the laboratory by opening them and removing the test swabs as aseptically as possible. The swabs were placed in tubes of nutrient broth and, sometimes, Brewer's thioglycollate broth, which were incubated at 37°C. for seven days, inspected, and plated out on blood agar for aerobic and some for anaerobic culture. The operation of transferring swabs to the bottles of broth, although carried out as aseptically as possible with sterile forceps, inevitably carries some risk of contamination (Pulvertaft, 1937; Savage, 1940). To measure the frequency of laboratory contamination, negative controls were always put through. These consisted of tins containing test swabs which were sterilized in hot air immediately before the dressing packs were due to be opened. In order to equalize the chances of contamination as far as possible, the transfer of control swabs to broth was sometimes done before, sometimes after, and occasionally during, the transfer of the test swabs.

Of 518 control swabs transferred to broth, 20 gave positive cultures, a blank contamination rate of 3·86%. The bacteriological efficiency of any packing method was measured by the degree to which its contamination rate exceeded this 'blank' figure.

The containers tested were cylindrical dressing drums, rectangular metal caskets with filter inserts in the lid and base and a gasket seal between the lid and body of the casket (G.U. Sterilizers Ltd.), and Bripac cartons.

The fabric wrapping materials, which were also sometimes used as liners in the other containers, consisted of unbleached calico and balloon cloth. The properties of these fabrics, determined by the British Cotton Industry Research Association, are shown in Table I.

TABLE I

THE PROPERTIES OF UNBLEACHED CALICO AND BALLOON CLOTH*

	Unbleached Calico		*Balloon Cloth*	
	Warp	*Weft*	*Warp*	*Weft*
Weight per unit area (oz./yd.²)	6·3		3·5	
Threads per inch	61	63	97	33
Thickness at 1 lb./in.², ²/1000in.	20		10½	
Air permeability (ml./cm.²/sec./cm.)	8·0		4·1	
Equivalent pore radius (microns)	50		29	

* Kindly supplied by the British Cotton Industry Research Associ. ion

The papers tested were (1) heavyweight crêpe paper of substance weight 28·1 with a high tear and stretch strength (Sterilwrap, Bowater Scott Corporation Ltd.) and (2) bleached Kraft paper of substance weight 22·0 (Messrs. E. S. and A. Robinson Ltd.).

RESULTS

Tests with unlined containers showed that only the metal caskets with filter inserts and a washer seal gave satisfactory results. Cardboard cartons (Bripac), although not satisfactory without linings, had a much lower contamination rate than unlined drums (Table II).

TABLE II

CONTAMINATION RATES OF LINED AND UNLINED CARTONS AND METAL CONTAINERS

	Storage Period 12 to 17 Days		
	Total Swabs	*Contaminated Swabs*	*% Contaminated*
Round drums, unlined	85	17	20·0
Round drums with calico liners	91	3	3·3
Metal caskets with washer seal in lids	82	2	2·5
Cardboard cartons, unlined (Bripac)	198	15	7·6
Cardboard cartons (Bripac) with fitted bleached Kraft paper liners	139	6	4·3

(Blank control 3·86%)

Both of the lined containers (drums and cartons) were bacteriologically efficient, with contamination rates which did not differ significantly from the blank control (Table II).

Except for the muslin, the fabric and papers tested may be used in one or more layers for either dressing packs or as linings for rigid containers. It was therefore necessary to assess their efficiency as barriers against recontamination. The results are shown in Fig. 1, in which each point represents the contamination rates of at least 24 test swabs and usually many more. (Bleached Kraft, not included in Fig. 1 gave results not significantly different from crêpe paper.)

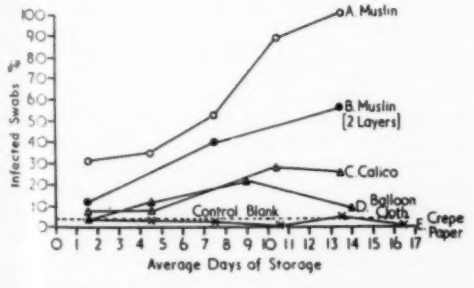
A. Muslin
B. Muslin (2 Layers)
C. Calico
D. Balloon Cloth
E. Crepe Paper
Control Blank
Infected Swabs %
Average Days of Storage
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
10 20 30 40 50 60 70 80 90 100

FIG. 1. *The contamination rates for crêpe paper and fabrics.*

As was to be expected, muslin was an inefficient barrier. Balloon cloth was better than calico but neither was as good as the paper, which even in a single layer, gave a contamination rate no higher than the blank figure. The few tests done with bleached Kraft paper showed that it was also more efficient than calico and balloon cloth. However, it was deficient in draping quality after it had been folded and autoclaved but was suitable for use as a liner or for making the paper bags in which small quantities of dressings are sometimes packed.

Although one layer of crêpe paper was bacteriologically efficient it should not be used routinely in a single layer because of the danger of puncturing or tearing during handling of the packages and damage from friction which may occur when many packages are handled together. Paper therefore should be used in double layers, and it will often be advantageous to enclose the packages so wrapped in rigid or semi-rigid containers, such as cardboard cartons, for added protection. The results of some packaging systems with paper and cardboard are shown in Table III.

TABLE III

CONTAMINATION RATES OF PAPER-WRAPPED PACKS WITH AND WITHOUT CARDBOARD CARTONS

	Storage Periods			
	8 to 13 Days		*14 to 21 Days*	
	Total Swabs	*Contaminated Swabs*	*Total Swabs*	*Contaminated Swabs*
One layer of heavyweight crêpe paper	62	1 (1·6%)	36	1 (2·8%)
The same in unlined cardboard cartons	172	5 (2·9%)	149	3 (2·0%)
The same in paper-lined cardboard cartons	118	0	229	7 (3·0%)

(Blank control 3·86%)

In these experiments, storage conditions were intentionally unsatisfactory. It may therefore be concluded that given good storage, the shelf life of packs wrapped in two layers of paper (and preferably enclosed in cartons for added protection) should be much longer than three weeks.

Although the paper and carton is highly efficient, it is rational to minimize the danger of recontamination by storing sterile packs under good conditions, *i.e.*, in dry cupboards free from dust and draughts. The packs should not be stored for long periods in open wards where airborne pathogens may abound.

The aerobic contaminating organisms in wards and corridors were investigated by tabulating the various species isolated from large numbers of packs of several kinds which had been exposed in the two situations (Table IV). Although most of the

TABLE IV

CLASSIFICATION OF AEROBIC CONTAMINANTS

	Packs Exposed in Surgical Wards	*Packs Exposed in Corridors*
Micrococci	141	126
Diphtheroids, *B. subtilis* type, moulds, etc.	63	110
Staph. aureus	7	0
Coliforms	5	9
Proteus and *Ps. pyocyanea*	2	0
Streptococci	4	0

organisms were non-pathogenic, appreciable numbers of pathogens were present in the wards; there were fewer pathogens in the corridors.

The risks that dressings stored in various parts of the hospital might be contaminated by anaerobic pathogens were investigated by culturing test swabs from muslin-wrapped packs stored in wards, corridors, and theatre (Table V). The frequency with

TABLE V

DISTRIBUTION OF NAGLER-POSITIVE CLOSTRIDIA ON SWABS IN TEST PACKS EXPOSED FOR AN AVERAGE OF 7·5 DAYS

	Total Swabs Exposed	*Positive Swabs*
Three surgical wards	73	35 (48%)
Ward dressing room	50	7 (14%)
Corridors and theatre	77	2 (3%)
Control swabs	32	0

which *Cl. welchii* was isolated in the wards was particularly striking. The principal sources were no doubt the large intestines of the patients and the main reservoir was probably their bedclothes. Several sweep plate cultures (Williams cited by Blowers and Wallace, 1955) show that *Cl. welchii* was often present in used blankets.

DISCUSSION

The ideal wrapping material for dressings and other sterile goods should readily allow steam to penetrate and should maintain sterility for long periods afterwards. It should be cheap, robust enough to withstand handling, convenient to use, and popular with the members of the nursing staff. The paper-wrapped packs inside paper-lined cardboard cartons possess these advantages. The rigidity and oblong shape of the cartons enables them to be easily handled in specially constructed carrying crates of perforated metal (Brig. J. D. Welch, personal communication). With this system, one person can carry more dressings than can be carried by two people using drums (Fig. 2); this system is suitable for central sterile supply departments. The cartons are easy to stack on shelves. For carriage within the hospital a small covered truck may be used which should be reserved for the purpose of carrying sterile materials and preferably able to be opened for loading from the side.

FIG. 2. *Method of carrying cartons in crates and loading them into a truck opening from the side.*

Irrespective of the packaging method adopted, the steam for sterilization must be dry. This condition is particularly important when paper and cardboard are used. With dry steam, cartons remain serviceable for more than two dozen autoclavings.

The long shelf life of packs wrapped in paper, with or without cardboard, should be of great value in hospitals served by central sterile supply departments. The full shelf life of paper-wrapped packs has not been determined but the results of the present investigation show that detectable recontaminat on under good storage conditions would not occur for many weeks at least.

Other kinds of paper may prove to be as efficient bacteriologically and even more convenient in use than the crêpe paper used in these investigations. But a good paper evidently provides a better wrapping material than the fabrics commonly used for this purpose.

We thank Dr. W. A. Gillespie for his help and advice, Dr. E. M. Darmady and Brig. J. D. Welch for their encouragement, and the Trustees of the Nuffield Provincial Hospitals Trust for a grant towards the cost of this investigation.

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Disinfection of hospital blankets with synthetic phenolic compounds

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SYNOPSIS A cheap method by which hospital blankets may be effectively disinfected (approximately 3d. per blanket) is described. A recommendation is made that blankets from the patients' beds be divided into: 'socially dirty' blankets to be laundered, possibly at infrequent intervals; and 'socially clean' blankets to be disinfected frequently. The wide range of a synthetic phenolic compound is described. This substance is effective against all the common pathogenic bacteria in the presence of organic matter, anionic, or cationic detergents. Details are given of laboratory trials with this method of disinfection and of pilot trials at the Group hospital laundry. The recommended method is simpler and takes less time than ordinary washing.

During recent months numerous reports have drawn attention to the fact that hospital blankets rapidly become heavily contaminated with organisms during use and many of them may be pathogenic. It is not altogether certain how important this factor is in the general problem of cross-infection in wards (Marsh and Rodway, 1954; Perry, Siegel, Rammelkamp, Wannamaker, and Marple, 1957; Schwabacher, Salsbury, and Fincham, 1958; Gillespie, Simpson, and Tozer, 1958) but it is clearly desirable that each newly admitted patient should be supplied with fresh, germ-free blankets, if this is reasonably practicable (Gillespie and Robinson, 1959).

For the hospital authority wishing to achieve this state of affairs much advice is now available (King Edward's Hospital Fund for London, 1959), although many of the recommendations can be adopted only at considerable expense, and others involve alterations in laundry procedure which may not be easy to bring about, particularly where the laundry is an independent organization and has to consider other users (Central Health Services Council, 1959).

This paper describes a method by which hospital blankets may be effectively disinfected at a low cost, with no additional expenditure for staff or equipment and with a minimum of disturbance to laundry procedure.

EARLIER RECOMMENDATIONS

RECOMMENDATION 1 All woollen blankets should be replaced by some form of cotton blanket which can be frequently boiled without deterioration (Oxford Regional Hospital Board, 1958). There are, however, objections to the precipitate adoption of this policy, though it may prove to be a satisfactory long-term one. The cost of equipping an entire hospital with cotton blankets and discarding existing stocks of woollen ones may be considerable. Preliminary reports on the 'appeal' of cotton blankets in terms of weight, warmth, texture, and wearing properties have been quite favourable but they can hardly be described as yet as a well-tried commodity. One of the expected advantages of cotton blankets was to cut down the dissemination of fluff but recent experiments lead one to suppose that the reverse may in fact be the case (Pressley, 1958). We, indeed, have repeated Pressley's experiments in full and obtained similar results.

RECOMMENDATION 2 Woollen blankets should be sterilized by boiling. Particular attention must be paid to procedure and the *p*H of the wash liquor to prevent shrinkage and felting (C.S.I.R.O. Wool Research Laboratories, 1959; British Launderers' Research Association and International Wool Secretariat, 1959).

In seeking to introduce this procedure one tends to encounter the disinclination of the laundry to change established practice. Furthermore, any error or carelessness may appreciably damage the blankets.

RECOMMENDATION 3 Blankets should be disinfected by dipping them in a solution of one of the quarternary ammonium disinfectants (Blowers and

Wallace, 1955; Gillespie *et al.*, 1958). For this method to be effective special attention must be paid to the washing procedure used at the laundry as the quarternary ammonium compounds are inactivated by anionic detergents and soap. A further disadvantage is that they are not very effective against *Ps. pyocyanea* and *M. tuberculosis*.

RECOMMENDATION 4 Blankets should be disinfected by means of formalin vapour (Caplan, 1959). The usual difficulty to be expected here is to find the staff and autoclave space to carry out the procedure.

RECOMMENDATION 5 Blankets should be sterilized by means of ethylene oxide (Thomas, West, and Besser, 1959). Once again the problem is finding suitable staff and apparatus.

RECOMMENDATION 6 Blankets should be disinfected by means of certain synthetic phenolic compounds (Ravenholt, Baker, Wysham, and Giedt, 1958). The compounds are inexpensive, they are not inactivated by soaps or detergents, and, therefore, can be used in association with ordinary laundry routine and those of choice are effective against all common pathogenic bacteria, including *Ps. pyocyanea* and *M. tuberculosis*.

Experiments conducted at this hospital have shown that simply immersing blankets in a ½% solution of Hycolin (a mixture of halogenated alkyl/aryl phenols) at 45°C. for 10 minutes with subsequent drying in air or in the Lister dryer effectively disinfects hospital blankets without causing appreciable felting, shrinkage, staining, or other deterioration and without leaving any appreciable smell on the blankets.

It is recommended that ward blankets should be divided into two groups: (1) those which are dirty and need washing to make them 'socially' clean. These can be sent to the laundry for the usual washing procedure at fairly infrequent intervals; (2) those which are 'socially' clean but need disinfecting for each new patient. These can be sent to the laundry for simple 'dipping' in disinfectant and drying. This causes minimum wear and tear on the blanket.

The following preliminary information was kindly supplied by the manufacturers[1]

DESCRIPTION AND PHYSICAL PROPERTIES OF HYCOLIN

Hycolin is a clear, golden-brown fluid stable at all temperatures between 0°C. and 100°C.; it does not deteriorate when stored over long periods. It has an odour which is mildly phenolic/alcoholic. Dilutions of Hycolin, even in hard or brackish water, are crystal clear to slightly opalescent and free from scum; they are very mildly alkaline, concentrations of 1%, 2%, and 5% in tap water having *p*H values of 8·1, 8·4, and 8·6 respectively.

In useful dilutions Hycolin is not escharotic or irritating to the skin and mucous membrane, and is inert to such substances as rubber, plastic, paint, fabrics, etc.

Hycolin is composed of a number of phenol derivatives incorporated in such a manner in a base of alcohol and anionic wetting agent as to give the widest possible spectrum of biocidal activity, covering a range which includes *Staphylococcus pyogenes*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*, together with *Mycobacterium tuberculosis* and pathogenic fungi such as *Trichophyton interdigitale* and *Candida albicans*.

SENSITIVITY TESTS Patch testing of 20 persons for periods of 24 and 48 hours failed to produce any reaction to 0·5% or 1% Hycolin in aqueous solution. A daily application of 0·5% aqueous Hycolin to the eyes of rabbits over a period of 14 days caused no irritation. Vaginal douching with 0·5% aqueous Hycolin produced no untoward effect.

BACTERIOLOGICAL TESTS All tests were performed at 20°C. Dilutions of Hycolin were made in tap water of 24° hardness (Clarks). Suitable controls were incorporated in each test to check the viability of the test organism.

SUSPENSION TESTS In test 1, 0·5 ml. of a suspension of the test organism was added to 5 ml. of Hycolin dilution. Subcultures were made at the appropriate time intervals into nutrient broth and incubated at 37°C. up to 10 days.

Hycolin Concentration	*Test Organism*	*Subculture after*			
		2½ min.	*5 min.*	*7½ min.*	*10 min.*
0·1%	*E. coli*	+	+	+	+
	Staph. pyogenes	+	+	+	+
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	+	+	+	+
0·5%	*E. coli*	–	–	–	–
	Staph. pyogenes	–	–	–	–
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	–	–	–	–

For tests 2 and 3 (which gave identical results), the same method was used as in test 1, the suspension being added to Hycolin diluted with either 20% aqueous blood serum or 20% aqueous skimmed milk.

Hycolin Concentration	*Test Organism*	*Subculture after*			
		2½ min.	*5 min.*	*7½ min.*	*10 min.*
1·0%	*E. coli*	+	–	–	–
	Staph. pyogenes	+	+	+	+
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	+	–	–	–
2·0%	*E. coli*	–	–	–	–
	Staph. pyogenes	–	–	–	–
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	–	–	–	–

[1] Messrs William Pearson Ltd., 172 Buckingham Palace Road, London, S.W.1.

Similar tests in the presence of soap and other inhibitors, such as thioglycollate, asparagin, primary phosphate, and Tween 80, namely, substances known to interfere with the efficiency of other types of germicide, showed Hycolin to be little affected. Concentrations ranging between 0·5% and 2·0% were sufficient to kill the test organisms in less than two-and-a-half minutes.

For test 4, 0·5 ml. of a culture of *M. tuberculosis* (H37RV) in Dubos medium was added to 5 ml. of Hycolin dilution. Subcultures were made at the appropriate time intervals into Dubos medium and incubated at 37°C. for four weeks.

Hycolin Concentration	Test Organisms	Subculture after			
		2½ min.	5 min.	7½ min.	10 min.
1·0%	*M. tuberculosis*	+	–	–	–
2·0%	*M. tuberculosis*	–	–	–	–

In test 5, to test the disinfection of fabrics, linen strips were soaked in a suspension of the test organism and dried at 37°C. for four hours. After immersion in Hycolin dilutions for various times the strips were rinsed in sterile water and subcultured at 37°C. in nutrient broth.

Hycolin Concentration	Test Organism	Subculture after			
		15 min.	30 min.	60 min.	2 hr.
1·0%	*E. coli*	+	+	+	–
	Staph. pyogenes	+	+	–	+
	Proteus vulgaris	+	–	–	–
	Ps. aeruginosa	+	+	–	–
2·0%	*E. coli*	–	–	–	–
	Staph. pyogenes	+	+	–	–
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	–	–	–	–
3·0%	*E. coli*	+	–	–	–
	Staph. pyogenes	+	–	–	–
	Proteus vulgaris	+	–	–	–
	Ps. aeruginosa	–	–	–	–

Test 6 was an extension of test 5, and the method was identical with that of test 5 apart from the Hycolin dilutions which were made in 1% linseed oil soap solution.

Hycolin Concentration	Test Organism	Subculture after			
		15 min.	30 min.	60 min.	2 hr.
2·0%	*E. coli*	+	–	–	–
	Staph. pyogenes	+	+	+	–
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	+	–	–	–
3·0%	*E. coli*	–	–	–	–
	Staph. pyogenes	+	+	–	–
	Proteus vulgaris	–	–	–	–
	Ps. aeruginosa	–	–	–	–

MYCOLOGICAL TESTS Two tests were carried out.

In test 7, from well-grown petri-dish cultures of the fungi, circles of 1 cm. diameter of the growth were cut with a sterile cork borer and immersed in Hycolin dilutions for various times. Before transplanting, the circles were rinsed with sterile water to remove excess Hycolin. Incubation at 22°C. was maintained for six weeks.

Hycolin Concentration	Test Organism	Subculture after		
		5 min.	15 min.	60 min.
2·0%	*Trich. interdigitale*	+	–	–
	Trich. acuminatum	–	–	–
	Candida albicans	+	+	–

In test 8, suspensions of the fungi in water were prepared by shaking the growth with broken glass. Sterile match sticks were soaked in the suspension and allowed to dry before being placed in Hycolin dilutions. After appropriate intervals the match sticks were removed from the Hycolin, washed, and transplanted on to Sabouraud's medium.

Hycolin Concentration	Test Organism	Subculture after		
		5 min.	15 min.	60 min.
2·0%	*Trich. interdigitale*	–	–	–
	Trich. acuminatum	–	–	–
	Candida albicans	–	–	–

SAMPLING BACTERIAL FLORA ON BLANKETS AT OLDCHURCH HOSPITAL

The method chosen to sample the bacterial flora was the sweep plate technique as used by Blowers and Wallace (1955). The following media were used at various times: 10% horse blood nutrient agar, Mannitol-salt agar, phenolphthalein phosphate agar, and C.A.T.B. agar.

Of these media the blood agar gave the highest total flora. There is no known specific inhibitory substance for phenol which can be incorporated into culture media, but tests carried out in this laboratory showed that Hycolin-treated blanket fibres inhibited the growth of pre-seeded *Staphylococcus pyogenes* (Heatley strain) on plain nutrient agar. No such inhibition could be detected when the staphylococcus was seeded onto 10% horse blood agar. Only occasional colonies of *Staph. pyogenes* were seen on the sweep plates taken from the blankets awaiting either laundering or treatment with Hycolin. This is consistent with the relatively low rate of staphylococcal cross-infection existing in this hospital at the moment. No colonies of this organism were seen on the plates after their treatment by either process. No colonies of *Pseudomonas aeruginosa* were detected.

The organisms grown fell into three groups:

(1) *Bacillus sp.*: aerobic, spore-bearing bacilli, sometimes produced large spreading colonies which tended to obliterate the other organisms.

(2) Micrococci: spherical, Gram-positive organisms gave opaque, butyrous colonies with white or coloured pigments. They showed a marked tolerance for 7% NaCl but were all plasma coagulase negative. Probably more than one genus was present.

(3) Coliform organisms is a term of convenience to describe a heterogeneous group of Gram-negative bacilli, including *Escherichia* sp., *Aerobacter* sp., *Chromobacterium* sp., *Achromobacterium* sp. No colony in this group resembled *Ps. aeruginosa.*

The blood agar plates were incubated at 37°C. for 24 hours and remained at room temperature for a further 48 hours before the final assessment of bacterial flora was made.

The selective media were incubated at 37°C. for 48 hours and then left at room temperature for a further 24 hours.

LABORATORY EXPERIMENTS Three blankets were taken from a surgical ward blanket store. Sweep plate cultures were taken from them. The blankets were then immersed in a ¼% solution of Hycolin for 10 minutes at 50°C. The excess fluid was removed with a wringer and the blankets hung overnight to dry in a part of the laboratory remote from the bacteriology department. When the blankets were dry, further cultures were taken from them and the blankets were returned for use on the ward. After one to two weeks on the patients' beds, the blankets were again sampled for their bacterial flora. The whole process was also carried out on three blankets taken directly from the patients' beds, instead of from the blanket store.

	No. of Colonies before Treatment	*Type of Growth*	*No. of Colonies after Treatment*	*Type of Growth*	*No. of Colonies after Use*	
Blankets from store						
1	150	Mixed flora	4	Bacillus	85	Mixed flora
2	15	Mixed flora	8	Mixed flora	400	Mixed flora
3	23	Mixed flora	13	Mixed flora	450	Mixed flora
Blankets from patients' beds						
4	132	Mixed flora	4	Bacillus	300	Mixed flora
5	150	Mixed flora	6	Bacillus	350	Mixed flora
6	350	Mixed flora	2	Bacillus	300	Mixed flora

These results showed that (1) the rather harsh laundry treatment used at this hospital removed a large number of organisms from the blankets, but the blankets quickly deteriorated on frequent laundering; (2) treatment with Hycolin substantially reduced the bacterial infection of the blankets; (3) the blankets quickly acquired a heavy bacterial flora after they were returned to the ward.

To test whether the synthetic phenols used were photosensitive a portion of new blanket was divided and one piece was immersed in Hycolin, dried, re-immersed, and dried. This process was continued for 12 immersions. The blanket hung indoors, in daylight, during this test. No difference in colour was perceptible between this piece of blanket and the control. After 10 dips in Hycolin, one face of the test piece of blanket was exposed to approximately 500 microwatts/sec./cm.^{2} of ultraviolet light for 24 hours. The blanket became yellowish-brown but a similar colour change of equal intensity took place with a control piece of blanket that had not been treated with Hycolin.

LAUNDRY TRIALS As the laboratory experiment results seemed encouraging, larger trials were carried out in the group laundry. Twenty-two blankets, sent from the hospital for laundering, were cultured with blood agar 'sweep' plates. The blankets were then immersed in 60 gallons of ¼% Hycolin at 45°C. in a Tullis washing machine. The machine was rotated once or twice to wet the blankets thoroughly and they were allowed to soak in the fluid for 10 minutes. They were then taken out, hydroed in the usual manner and dried in a Lister hot air drier for 20 minutes at 280°F. When dry, sweep plate cultures were again taken (Result 1).

A further 22 blankets were similarly treated but after being hydroed they were hung outside the laundry for five or six hours to dry, with many other blankets, this being the normal process of drying blankets at this laundry in the fine weather (Result 2).

	No. of Colonies before Treatment		*Type of Growth*	*No. of Colonies after Treatment*		*Type of Growth*
	Range	Average		Range	Average	
1	500-30	174	Mixed flora	18-2	7	*Bacillus* sp.
2	500-50	190	Mixed flora	100-4	17	*Bacillus* sp.

These results suggested that the Lister dried blankets gave better results for the following reasons:

The hot air in the drier alone may have had a sterilizing effect.

The blankets were subjected to hot phenolic vapour while drying.

The blankets hanging in the open air may have become recontaminated during their drying period.

Further tests were carried out by selecting 12 blankets that had been laundered in the normal way and hydroed. The blankets were then divided into two groups of six. One group was dried in the Lister hot air drier and the other group dried in the open air. When dry the blankets were sampled with blood agar sweep plates. It was assumed that these blankets carried the same number of organisms, before laundering, as those tested previously, *i.e.*, 180 colonies.

	No. of Colonies Present		*Type of Growth*
	Range	Average	
Blankets dried in Lister dryer	80-8	38	Mixed flora
Blankets dried in open air	200-100	160	Mixed flora

In both groups of blankets the resulting bacterial flora was of a mixed variety which could include staphylococci in certain circumstances.

The hot phenolic vapour must therefore have a more inhibitory effect than hot air alone, since only a few spore-bearing bacilli were left on the Hycolin-treated blankets after this method of drying, so Hycolin-treated blankets should be dried in a hot-air drier for the best results.

To meet the point made by Blowers and Wallace (1955) that the residual antiseptic on the blanket after dipping must be neutralized, to avoid possible misleading effects of bacteriostasis rather than lethal effects, some further tests had to be carried out. Hycolin has no known specific neutralizing agent acting at high dilution. It was therefore necessary to remove the antiseptic by steeping the blanket in water for 24 hours after dipping.

The following testing routine was undertaken:

(1) Sweep cultures were carried out on used blankets. These produced the usual concentrations of flora.

(2) The blankets were dipped in Hycolin for 10 minutes at 5°C.

(3) Dried in air.

(4) Sweep plate tests were made. These showed the previously observed reduction of, or elimination of, organisms.

(5) The blankets were steeped in plain water for 24 hours.

(6) Dried in air.

(7) Sweep plates showed no increase in flora when compared with (4) above, and no inhibition of *Staph. pyogenes* (Heatley strain) on a pre-seeded nutrient agar plate was detected (*vide supra*). Two further experiments were carried out with *M. tuberculosis* and *Pseudomonas aeruginosa* (*pyocyanea*) involving the artificial inoculation of these organisms onto blankets.

M. TUBERCULOSIS Four small portions of blanket were sterilized and heavily inoculated with 21-day-old liquid Dubos culture of *M. tuberculosis* H 37 RV.

Two pieces of blanket were treated with ½% Hycolin at 45°C. for 10 minutes, rinsed in water, and allowed to drain. Two control pieces were rinsed in water and allowed to drain. Each of the four pieces of blanket was pressed onto Dubos agar medium and discarded, and the culture plates were sealed and inoculated at 37°C. After 21 days' incubation the culture plates from the control blankets showed many colonies of *M. tuberculosis* while the culture plates from the Hycolin-treated blankets remained sterile.

PS. AERUGINOSA (PYOCYANEA) A freshly isolated strain of *Pseudomonas* was grown for 24 hours in 250 ml. of nutrient broth. The whole volume of culture fluid was emptied into 5 gallons of water, thoroughly mixed, and a hospital blanket totally immersed. The excess fluid was wrung out and the blanket dried overnight.

A sweep plate culture of the dried blanket showed 120 colonies of *Ps. aeruginosa* on blood agar. The blanket was then treated with Hycolin in the same manner as previously outlined in the laboratory procedure.

When dry, sweep-plate cultures showed no growth of *Pseudomonas*.

These experiments showed that the recommended concentration of Hycolin will destroy relatively large numbers of tubercle bacilli and *Pseudomonas* on woollen blankets.

We are grateful for the facilities granted to us by Mr. Evans, Secretary, St. George's Hospital, Hornchurch, at the hospital group laundry. Without the help and cooperation of Mrs. Pratt, laundry manageress, this work could not have gone forward.

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Skin disinfection

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Although skin disinfection has been a subject of interest and research for over 100 years, there is no generally accepted procedure for use either at the operation site or on the hands of surgeons and nurses. This state of affairs is understandable, for new antiseptics continue to appear, and the information which clinicians have had from laboratory study on their relative and absolute merits is far from clear. Depending on whether they were testing the effects of antiseptics on the superficial or the deeper flora of the skin, bacteriologists have stressed either the 'virtual disinfection' by some antiseptics or the impossibility of removing more than a fraction of the skin flora without destroying the skin. Moreover, many discrepancies in the evaluation of individual antiseptics have been due to differences (and often deficiencies) in the technique of testing.

THE PURPOSES OF SKIN DISINFECTION

In hospital practice the removal of bacteria from the skin is desired essentially for two reasons: (1) To prevent cross infection by blocking the transfer of pathogenic bacteria from the hands of nurses and doctors to the susceptible tissues of patients; and (2) to prevent self-infection of patients by blocking the transfer of pathogens from the skin to the underlying tissues on a knife blade or a needle. In the case of the surgeon's or the nurse's hands, the object is to remove or reduce the numbers of pathogens on the surface of the skin, and to achieve a cumulative effect by repeated application of the antiseptic. At operation sites, on the other hand, it is desirable that pathogens should be removed from the deeper layers as well as from the surface of the skin, and often there will be no opportunity for more than one application of the antiseptic. In addition to the removal of vegetative organisms, preparation of the operation site and hand cleansing involve the removal from the exposed skin and from under finger nails of chemical dirt, grease, and the spores of pathogenic clostridia which cannot be destroyed by short exposure to any antiseptic. All these factors must be considered in defining the requirements for a technique of skin disinfection.

BACTERIAL FLORA OF THE SKIN

The healthy skin may be regarded as a selective medium. Certain pathogens, *e.g.*, *Streptococcus pyogenes*, are killed by unsaturated fatty acids of the sebaceous secretions, and others, such as the Gram-negative bacilli, are to a large extent killed when the fluid medium in which they were deposited on the skin evaporates (Burtenshaw, 1942; Ricketts, Squire, and Topley, 1951). The bacteria relatively unaffected by these factors which can multiply on the skin fall mainly into three groups: (1) Micrococci, (2) corynebacteria, and (3) *Propionobacterium acnes*, an anaerobe which Evans, Smith, Johnston, and Giblett (1950) have found to be the predominant organism (see also Pillsbury and Kligman, 1954). These groups of bacteria are the 'resident' flora defined by Price (1938, 1957) as the stable bacterial population of the skin which lives and multiplies there, in the depths and hair follicles as well as on the surface. Resident bacteria appear to be for the most part harmless commensals, but they sometimes include *Staphylococcus aureus*. What Price describes as the 'transient' flora includes any bacteria which are deposited on the skin from the environment; in hospitals these will include a variety of potential pathogens.

THE ASSESSMENT OF SKIN DISINFECTION

The methods used to assess the value of skin disinfection have been mainly concerned with four criteria: (1) The incidence of wound sepsis; (2) the removal of the superficial flora, represented by cultures deposited on the skin; (3) the removal of resident as well as transient flora, shown by bacterial counts in standard hand-scrubbing tests; and (4) the bacteria in skin biopsies taken after pre-operative disinfection.

The first of these criteria is clearly the most valuable when it can be applied successfully, but its scope has been very limited so far. The classic example is the demonstration by Semmelweiss in 1847 of a dramatic fall in the death rate from puerperal sepsis in maternity wards after the introduction of routine hand disinfection with chlorinated

lime by doctors and midwives (see Sinclair, 1909). A recent example is the fall in staphylococcal infection of the newborn following the introduction of hexachlorophene emulsion (Farquharson, Penny, Edwards, and Barr, 1952; Hill, Butler, and Laver, 1959). But when, as in these studies, the initial incidence of infection is small or highly variable, it is difficult to assess the value of skin disinfection by a fall in the sepsis rate. Clinical records of wound 'sepsis' are notoriously variable, and reports (*e.g.*, Kramer and Sedwitz, 1944) on the virtual elimination of sepsis in clean operation wounds following the introduction of a particular antiseptic provide little evidence of its value unless special techniques of recording are used.

Colebrook and Maxted (1933), Gardner and Seddon (1946), and Gardner (1948) allowed bacterial cultures to dry on the skin and then treated the inoculated areas with antiseptics; estimates were made of the bacteria which survived exposure to the antiseptics. Story (1952) developed this method, using a penicillin-resistant staphylococcus as the test organism and a selective medium containing penicillin. Bacteriostatic controls or neutralizers were used to avoid erroneous results through the transfer of antiseptic to culture plates. By the use of these methods of testing, 'virtual disinfection', *i.e.*, destruction of at least 99·9% of the organisms applied, was obtained in about 20 seconds when the surface was treated with 2% iodine in 70% ethyl alcohol. The effectiveness of an antiseptic was estimated by the time it took to achieve virtual disinfection, and by the proportion of samples in which this effect was obtained.

Price's test for the resident flora (Price, 1938, 1957) involves a standard one minute scrub with soap and water in a series of eight basins before and in a second series of eight basins after treatment with antiseptic followed by neutralizer. Simpler tests for the resident flora have been devised by Cade (1950), Quinn, Voss, and Whitehouse (1954), and Bowers (1950). Price (1951) introduced a 'spot testing' method to study the bacterial flora of different areas of the body. The proportion of resident bacteria shown in such tests to be removed by antiseptics falls considerably short of virtual disinfection; *e.g.*, after two minutes' application of 2% iodine in alcohol, there was a 95% reduction in the flora shown by the serial basin test.

The culture of skin biopsies taken after preoperative disinfection has been used for the assessment of skin antiseptics by Walter (1938), Gardner and Seddon (1946), Murphy, Dull, Gamble, Fultz, Kretzler, Ellis, Nichols, Kucharczuk, and Zintel (1951) and Myers, MacKenzie, and Ward (1956). This method has the virtue of providing a sample of bacteria from all levels of the skin, but it is awkward to use and for quantitative assessments should include the excision of skin from untreated as well as from disinfected areas. A surprisingly large proportion (23/24) of such biopsies was found to be free from detectable bacteria after four minutes' pre-operative treatment with 0·5% aqueous chlorhexidine (Myers *et al.*, 1956). This might cast some doubt on the view that the deeper layers of the skin retain their flora after disinfection; but as the biopsies were small fragments of tissue, some of which yielded no bacteria even before the antiseptic was applied, the organisms removed may in most cases have been superficial or transient flora, deeper resident bacteria being absent from the samples examined.

ANTISEPTICS USED FOR DISINFECTING THE SKIN

The chief properties required in an antiseptic for operation sites are (1) that it should be rapidly active against a wide range of microorganisms (ideally against all pathogens); (2) that it should kill the organisms and not merely inhibit their growth; and (3) that it should not damage the skin or the underlying tissues either by direct toxic action or by sensitization. Other properties, such as penetration and temporary staining of the skin to show the disinfected area, may be desirable. Antiseptics used for regular disinfection of the hands need not be as rapid in their action as those used on operation sites, and it is an advantage if they leave a film on the skin which continues to inhibit the growth of bacteria.

Of the wide range of antiseptics which have been used for skin disinfection, many do not fulfil these requirements. Ordinary soaps, which have a limited activity against some bacteria, are inactive against staphylococci (Bayliss, 1936). Phenol, which was used by Lister (1875) in a 5% aqueous solution, was later discarded because of its toxicity and because other antiseptics were active in much lower concentrations. Among the latter were many compounds of mercury, including mercurochrome, merthiolate, and the phenyl mercuric salts, but these antiseptics are predominantly bacteriostatic (Geppert, 1889; Hoyt, Fisk, and Burde, 1942). The great vogue of the mercurials has undoubtedly been stimulated by deceptive tests in which inhibitory concentrations of the antiseptic were carried over to culture plates. The acridine dyes (Albert, 1951) are strongly bacteriostatic, but their bactericidal action is slow and selective; the Gram-negative bacilli (in particular *Ps. pyocyanea*) are relatively insensitive to these compounds. Alcoholic solutions of acridines and mercurials are often used, but the former apparently owe the whole of their dis

infectant powers to the solvent (Gardner and Seddon, 1946), and mercurials have been found actually to reduce the skin disinfecting powers of alcohol (Price, 1957). Colebrook and Maxted (1933) showed that chloroxylenol has outstanding merit as a hand disinfectant in midwifery because of its action against *Strep. pyogenes*, but this compound is deficient in activity against the Gram-negative bacilli, and in particular against *Ps. pyocyanea* (Lowbury, 1951).

Iodine was recommended by Senn as a skin disinfectant in 1905, and ethyl alcohol was used still earlier for this purpose (Epstein, 1897). Both have a rapid bactericidal action against a wide spectrum of vegetative organisms. Ethyl alcohol has optimal skin disinfecting power when diluted with water, preferably at concentrations between 70 and 90% (Epstein, 1897; Price, 1957). Colebrook and Maxted (1933) found an aqueous solution of iodine to be an effective bactericide for the hands with a prolonged action after drying. Unfortunately, iodine causes severe sensitivity reaction in some patients (*e.g.*, Murphy *et al.*, 1951; Zintel, 1956) and has for this reason been avoided by many surgeons. Recently, mixtures of iodine with certain surface active agents (iodophors) have been found to be free from the staining and sensitizing effects while retaining the disinfectant power of aqueous or alcoholic iodine (Gershenfeld and Witlin, 1955); of these antiseptics Virac has been specifically recommended for skin disinfection (Frisch, Davies, and Krippaehne, 1958).

The quarternary ammonium compounds have been a popular choice for skin disinfection because in addition to their disinfectant powers they are good detergents. Price (1950) found an additive effect of the two antiseptics when alcoholic solutions of benzalkonium chloride were used for skin disinfection. The value of aqueous solutions of such compounds is more doubtful. Like the mercurials, quaternary compounds are apt to give misleading results in tests of skin disinfection through the transfer to culture medium of bacteriostatic concentrations which do not kill bacteria (Neufeld, 1943). Moreover, a film of the antiseptic may be left on the skin concealing viable organisms (Miller, Abrams, Huber, and Klein, 1943). A potential disadvantage of these compounds is their incompatibility with soap, traces of which may be present on the skin and interfere with disinfection by a quarternary compound.

Of the antiseptics introduced in recent years two are of special interest for their role in skin disinfection. Hexachlorophene or G.11, a bis-phenol, was first described by Traub, Newhall, and Fuller (1944); its activity primarily against Gram-positive organisms, its compatibility with soap, its slow but cumulative action through deposition as a film on the skin, and its lack of toxic or sensitizing properties make it very suitable for regular use as a hand disinfectant by surgeons and nurses. At a concentration of 2% in bar soap, or at 3% in a cream containing an anionic detergent *p*HisoHex, hexachlorophene is found to be a useful agent for this purpose (Seastone, 1947; Hufnagel, Walter, and Howard, 1948; Smylie, Webster, and Bruce, 1959). Chlorhexidine (Hibitane) has been reported to have value, in aqueous or alcoholic solution, as an antiseptic for the operation field (Myers *et al.*, 1956), and also as a hand disinfectant applied in a cream by nurses (Murray and Calman, 1955; Calman and Murray, 1956).

At the Birmingham Accident Hospital we have recently investigated the relative merits of these and of some other antiseptics for the disinfection of hands and of operation sites (Lowbury and Lilly, 1960; Lowbury, Lilly, and Bull, 1960). The essentials of this study are summarized in the following sections.

THE SURGEON'S HANDS

Devenish and Miles (1939) found up to 24% of rubber gloves punctured at the end of operations. Large numbers of bacteria could easily be transferred to an operation wound through such holes and also through wet patches on the sleeves of theatre gowns.

We compared a number of alternative measures by which these hazards might be reduced (Table I). The tests were made on five members of the laboratory staff. After scrubbing up with soap and water (control series) or scrubbing up and, in addition, using some form of disinfection, these operators wore rubber gloves for an hour, one glove in each pair having a small pin hole at the tip of each finger. After this mock operation, the gloved hands were washed and dried, and samples were taken from which estimates were made of the bacteria that emerged through holes in the gloves and of the bacteria left inside the gloves after use. Appropriate neutralizers and bacteriostatic controls were used.

There was a substantial reduction in the numbers of bacteria escaping through holes in the gloves and deposited inside the gloves after the use of *p*HisoHex for the scrub and for all ablutions in the previous week, and a smaller reduction after a similar use of hexachlorophene soap. Very good results were also obtained after a three-minute rinse with 70% ethyl alcohol (slightly improved by the addition of 0·5% chlorhexidine digluconate), and by the addition to glove powder of neomycin and bacitracin (5 mg. per gram of each); a smaller effect

TABLE I

EFFECT OF ANTISEPTIC TREATMENT ON FLORA OF HANDS AFTER ONE HOUR'S OPERATION

Antiseptic Treatment of Hands	*Mean Bacterial Counts (per ml.) on Glove Washings after*			*Mean Estimated Counts (per ml.) on Washings of Bacteria Emerging through Holes in Gloves after*		
	(1) *Scrubbing up + Antiseptic Treatment* (10 *Samples*)	(2) *Scrubbing up Only (Controls)* (10 *Samples*)	(3) (1) *as %* *of* (2)	(1) *Scrubbing up + Antiseptic Treatment* (19-20 *Samples*)	(2) *Scrubbing up Only (Controls)* (18-20 *Samples*)	(3) (1) *as %* *of* (2)
Neomycin + bacitracin in glove powder	0·6 ± 1·1	332 ± 120	0·18	3·7 ± 3·1	73·6 ± 17	5·0
70% alcohol rinse (3 minutes)	3·0 ± 0·7	62·5 ± 18	4·8	7·5 ± 5·4	81·0 ± 12	9·2
70% alcohol with 0·5% chlorhexidine rinse (3 minutes)	3·1 ± 0·7	151 ± 48	2·1	3·25 ± 2·1	69·9 ± 14	4·6
Hexachlorophene soap (for 5-minute scrub and previous week)	14·9 ± 6	169 ± 66	8·8	7·3 ± 2·8	51·2 ± 8·7	14·3
*p*HisoHex (for two-minute scrub and previous week)	1·9 ± 0·7	127 ± 39	1·5	2·7 ± 1·3	63·8 ± 13	4·2
Spirit swab	79·5 ± 36	392 ± 140	20·3	24·2 ± 4·1	63·2 ± 14	38·3

was obtained after rapidly mopping and drying the hands with industrial spirit. In favour of hexachlorophene preparations are the facts that no additional time is needed for applying the antiseptic, and that the disinfection applies to the whole surface of the body; the risk of contamination through wet sleeves as well as through holes in gloves should therefore be reduced.

THE NURSE'S HANDS

For periods of 14 days, six nurses in each of three wards used ordinary soap, and then hexachlorophene soap for all ablutions, and finally ordinary soap again. At the end of each week viable counts for total organisms and for presumptive *Staph. aureus* were made on standard handwashings in Ringer's solution from each of the nurses.

Table II shows that the counts of total organisms and of presumptive staphylococci were reduced by about two-thirds during the period when hexachlorophene soap was being used. Whereas 86% of the samples from hands washed with ordinary soap yielded *Staph. aureus*, these organisms were isolated from only 46% of the samples from hands washed with hexachlorophene soap; if the Burns Unit, in which the risks of contamination are exceptional, was left out of the assessment, the use of hexachlorophene soap was associated with a reduction from 79% to 33% of samples yielding *Staph. aureus*.

TABLE II

EFFECT OF HEXACHLOROPHENE SOAP ON HAND FLORA OF NURSES

Soap used in Ward and Off Duty	*All Colonies*		Staph. aureus	
	Mean Count per 0·1 ml. Washings	*Samples*	*Mean Count per 1·0 ml. Washings*	*Samples*
Ordinary (1st fortnight)	504 ± 72	35	72·1 ± 13	36
Hexachlorophene (2nd fortnight)	186 ± 74	42	29·7 ± 8·5	42
Ordinary (3rd fortnight)	635 ± 74	27	127 ± 52	27

THE OPERATION SITE

Our chief aim was to find an alternative to alcoholic iodine which would be as effective in reducing the skin flora but free from the risks of sensitization and irritation. We tested some of the newer antiseptics, including chlorhexidine, Virac (an iodophor), and Penotrane (phenyl mercuric dinaphthyl methane sulphonate); hexachlorophene was not included, because its slow action makes it unsuitable for disinfection of the operation site. In subsidiary experiments we examined the elimination of *Staph. aureus* from hand carriers, and the effect of successive treatments with antiseptic.

In the first experiment we used a method similar to that of Story (1952) to assess the effect of antiseptics on staphylococcal cultures deposited and allowed to dry on the skin (Table III). Each of the antiseptics except aqueous Penotrane and cetrimide removed all detectable staphylococci from the surface of the skin during two minutes' exposure to the antiseptic.

In the second experiment (Table III) we examined the effect of antiseptics on the resident flora. Six antiseptics which had removed all detectable organisms in the first experiment were examined with controls by a standard handwashing technique on seven subjects. Viable counts were made of washings taken before and after two minutes' treatment with antiseptic or (in the controls) before and after a rinse in running water. A Latin square design was

TABLE III

COMPARISON OF EFFECTS OF DIFFERENT ANTISEPTICS ON SUPERFICIAL AND ON RESIDENT FLORA OF SKIN

Antiseptics	*Approximate Duration of Treatment (min.)*	*Superficial Organisms (culture of staphylococci) Applied to Skin*		*Resident Organisms*	
		Mean % Organisms Surviving after Treatment	*Tests*	*Mean % Organisms Surviving after Treatment*	*Tests**
None	—	—	—	85·3	7
Ethyl alcohol (70%)	0·4	0·008 – 1·2	4	—	—
	2·0	<0·0004	3	31·4	7
Iodine (1%) in ethyl alcohol (70%)	0·4	<0·012 – 0·24	7	—	—
Iodine (1%) in ethyl alcohol (70%)	2·0	—	—	19·3	7
Chlorhexidine (0·5%) in ethyl alcohol	0·4	0·002 – 0·03	2	—	—
Chlorhexidine (0·5%) in ethyl alcohol	2·0	—	—	18·7	7
Lugol's iodine	2·0	<0·0003	2	32·6	7
Virac	2·0	<0·0003	2	41·2	7
Chlorhexidine (0·5%) in water	2·0	<0·0003	2	39·3	7
Cetrimide (2%) in water	2·0	>0·09 – >0·14‡	2	—	—
Penotrane (0·1%) in water	2·0	6·4 – 12·2	4	(67·2)†	2

* Latin square design.
† Separate experiment, not included in Latin square design.
‡ Inhibition on culture plate not completely neutralized.

used for the experiment. Chlorhexidine (0·5%) in 70% ethyl alcohol was found to be as effective as 1% iodine in alcohol, and significantly more effective than any of the other methods tested; the best method reduced the skin flora by approximately 80%.

Nurses' hands were usually found to lose *Staph. aureus* in about the same proportions as they lost total organisms after disinfection with alcohol; in these subjects staphylococci were obviously carried with the resident rather than the transient flora. Attempts to improve the effectiveness of disinfection by prolonging the period of exposure to an antiseptic (ethyl alcohol) were disappointing; four minutes' treatment was sometimes more effective than two minutes' treatment, but a longer period of disinfection did not cause any further reduction in the bacterial flora, presumably because the remaining bacteria were too deeply placed to be reached by the antiseptic.

COMMENTS

The limited effect of antiseptics on the resident flora is disappointing. Some improvement can be expected by preliminary vigorous cleansing with detergents, *e.g.*, quarternaries, and fat solvents, and by the application of antiseptics with friction (Price, 1938); but the inaccessibility of the deeper flora to agents applied at the surface would seem to limit the possibilities of skin disinfection. This *impasse* might be overcome by the use of an antiseptic that penetrates to the deeper layers; a compound of this kind (Penotrane) was tested (see Goldberg, Shapero and Wilder, 1950), but in our study its effects were largely bacteriostatic. Fortunately *Staph. aureus* is usually absent or sparsely represented in the skin. Moreover, Elek and Conen (1957) have shown that a million or more virulent staphylococci are required for the initiation of a suppurating infection. If an antiseptic does nothing more than eliminate the transient flora and reduce the numbers of the resident skin staphylococci this may, nevertheless, prevent the development of sepsis by reducing the inoculum of staphylococci at the site of surgical incision or puncture.

Price (1938) has shown that the skin flora return gradually over a period of several days to the original level after effective disinfection. For this reason it should be possible to maintain the bacterial flora of the hands of nurse or surgeon at a low level without excessive rubbing when antiseptics such as hexachlorophene are used regularly. Apart from some drying of the skin, hexachlorophene appears to have little if any tendency to cause skin reactions. Sensitization to bacitracin also appears to be very rare (Jawetz, 1956) but skin reactions to neomycin are occasionally reported (Calnan and Sarkany, 1958).

Repeated disinfection on successive days is sometimes used for operation sites, especially in orthopaedic surgery, but it is doubtful if the level of colonization is lower after this procedure than it is after a single thorough disinfection on the operating table. Repeated cleansing with detergents is desirable before operations on skin containing ingrained dirt, and especially on the hands of gardeners and agricultural workers which may carry large numbers of clostridial spores; these cannot be destroyed by antiseptics or completely removed by

one cleansing with soap and water, but as they belong to the transient flora they are gradually shed from the skin with the horny layer.

I am grateful to the Editor of the *British Medical Journal* for permission to quote in this paper the results in the section on the disinfection of operation sites and hands.

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Efficiency tests on a series of common skin antiseptics under ward conditions

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SYNOPSIS Bacteriological trials were carried out on 11 different skin disinfecting agents to ascertain their relative efficiencies under ward conditions. A statistical analysis of the results is given. Of the antiseptics used, 5% Laurolinium appeared to be the most effective and has been adopted for routine use in the wards.

In 1958, a survey of sterilizing methods was made in two of the hospitals of the Portsmouth Group before the introduction of a central sterile supply.

It was found that 60% of the sterile procedures carried out in the wards were the giving of hypodermic injections and that the techniques and skin disinfectants used varied considerably from one ward to another. This paper describes the investigations carried out to determine the efficiency of skin disinfection before injection under ward conditions.

HISTORICAL

In the past many of the tests made to evaluate skin disinfection were of a laboratory nature. Fleming in 1924, using the Wright 'slidecell' method, illustrated the germicidal and leucocyte-inactivating properties of blood and used it for evaluating a number of antiseptics. Another approach was to study the effect of antiseptics on the skin of animals *in vivo* and on egg membranes (Browning, 1934; Hunt, 1937; Green and Birkeland, 1944). These tests, however, were of little practical value in assessing the effects of disinfectants on human skin.

In his book 'Disinfection and Sterilization,' Sykes (1958) described a direct swabbing technique which he had found satisfactory. This tested the retention of activity by disinfectants other than soaps and had been adapted from that described by Colebrook and Maxted (1933). A similar method was proposed by Story (1952) in which small circles of skin on the forearm were infected with a drop from a chosen culture. Various handwashing tests have been published by different workers devised primarily for testing antiseptic soaps and, of these, that described by Cade (1950) seems to be most generally used. None of these investigations gave any indication as to the number of bacterial colonies likely to be found on the skin of patients, nor of the ability of disinfectants under ward conditions to reduce their numbers. In addition they did not simulate the actual procedure carried out by a nurse when swabbing the arm of a patient before giving an injection. In view of the fact that the nurse swabs the area of skin once or twice at the most, it was felt important to determine whether any of the antiseptics used in this way were effective.

Eleven antiseptics were chosen for trial, since they appeared to be those most commonly used in the hospitals where the tests were carried out. These were: Roccal 1 in 40, methylated ether, cetrimide 0·5% with Hibitane, 0·05% Penotrane, 1 in 20 dequalinium chloride, 0·5% merthiolate in aqueous solution, 1 in 1,000 tinct. merthiolate, 1 in 1,000 with 50% alcohol, solution of iodine 1% in 90% spirit, and Laurolinium 1% and 5%.

METHOD

To determine the number of organisms on a given area of skin the technique of 'replica plating' was used (Lederberg and Lederberg, 1952). Replica plating was also used to provide a simple form of viable count by Elek and Hilson (1954). The advantages of this method are that a relatively large area of skin, 1 in. × 1 in., can be tested and the transfer carries over very little more antiseptic than the amount adhering to individual organisms. The replica blocks used consisted of 1 in. × 1 in. × 1 in. wooden blocks on one of whose surfaces was glued a square of velvet 1 in. × 1 in. This provided the 'contact' area. One of these blocks could be used many times and was easily sterilized by steam. The portion of skin used for the tests was the same for every patient, namely, an area on the inner aspect of the arm 2 in. below the cubital fossa. The culture media used were nutrient agar for methylated ether, 1% iodine in 90% spirit, and 70% alcohol, and blood agar for all the other disinfectants.

To carry out the trial, the block was first placed on the area B (shown below) of a culture medium plate to pick up a thin film of agar on its velvet surface. The block was then applied to the patient's arm to pick up bacteria and then pressed lightly back onto the same area of the plate. The arm was then swabbed quickly twice with a ball of sterile cotton wool dipped in the antiseptic solution being tested. Another identical sterile 'replica' block was taken and placed on square A of the agar plate. It was then pressed lightly first onto the patient's arm over the swabbed area and then onto the same square.

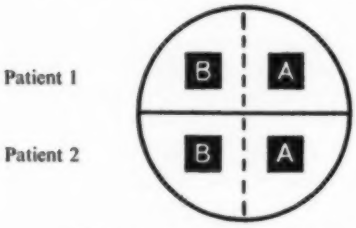

Diagram of an agar plate used in the test. One plate was used for each two patients.

This procedure was repeated on 100 different patients, male or female, for each solution under trial.

After 24 hours' incubation at 37°C. the plates were examined and the number of colonies of bacteria on each square counted.

Certain possibilities have to be considered when carrying out these tests. Since the area of skin used for each of the two samples was the same, difficulty might have been experienced when interpreting the results because the bacterial population might, in fact, have been reduced by the first sampling, and the apparent reduction in colony count be due to this factor and not to the action of the particular antiseptic solution used in the test. In order to exclude this possibility a further 100 tests were carried out in which the same skin area was sampled twice without any intervening swabbing with antiseptic solution.

A further possibility also had to be taken into account when interpreting the results and that was 'carry-over' of the antiseptic fluid to the culture plate. Should this happen the counts would not reflect truly the immediate disinfecting powers of the solutions. This applied particularly to the slower-drying aqueous solutions.

Supplementary investigations were, therefore, carried out on those solutions which were slower to volatilize by using culture medium to which a suitable inhibitor had been added, thus preventing continued action by any of the antiseptic carried over. These supplementary tests were carried out on both of the Laurolinium solutions and the cetrimide-Hibitane mixture, since from early results it appeared that these solutions had not completely evaporated before the skin was resampled after swabbing. The inhibitor used was 1% lubrol W with 0·5% lecithin.

For each of these tests four blocks and two blood agar plates, to one of which the inhibitor had been added, were used. Samples were taken simultaneously from two adjacent sites on the routine test area of the arm and plated out onto square B of the two different culture plates. Both skin areas were then swabbed with the test solution and a further two blocks applied to them and again pressed onto the two areas A of each plate.

RESULTS

It was found that taking two consecutive samples from the same area of untreated skin without intervening swabbing slightly reduced the number of colonies isolated from the second replica block when compared with the first, but the reduction was not statistically significant. The counts on inhibitor and non-inhibitor culture plates also showed no significant difference. These two possibilities can, therefore, be discounted when assessing the relative efficiency of the different disinfecting fluids.

The table summarizes the results of the plate counts before and after the application of the various fluids to the skin and gives their statistical significance.

Because the number of colonies counted showed such a wide range from 0 to more than 100, with the smaller counts predominating, the actual figures obtained were replaced by their square roots, as shown in column 3 of the table. These figures were used in the analysis. For example, if the number of colonies counted was 100 or more, the square root was taken as 10. This gave a more even distribution and made the statistical analysis more likely to be correct. The figures in column 3 show the mean decrease in terms of the square root of the number of colonies after swabbing. The antiseptic solutions are placed in the order of their apparent effectiveness.

DISCUSSION

The disinfecting agents fall into five groups, showing different degrees of effectiveness. Within the individual groups the solutions appear fairly uniform in their action.

The tests of significance given in columns 4 and 5 of the Table show that with the exception of Penotrane and aqueous merthiolate (group I) all the solutions had a significantly destructive effect on the skin flora. Methylated ether (group II), though better than the group I reagents, was relatively ineffective. Dequalinium chloride and 70% alcohol (group III) were significantly more potent than the group II solutions.

TABLE

RESULTS OF COMPARISON OF 11 SKIN DISINFECTING AGENTS

(1) *Skin Sterilizing Agents*	(2) *No. of Patients*	(3) *Mean Decrease in Square Root of No. of Colonies after Swabbing*	(4) *Statistical Significance of Decrease Compared with that for No Agent*	(5) *Statistical Significance of Decrease Compared with that for Last Preceding Agents*
GROUP I				
None (control test)	100	0·18	—	—
Penotrane stock solution 1:20	100	0·29	Not significant ($P > 0·10$)	—
Merthiolate (aqueous) 1 in 1,000	100	0·34	Not significant ($P > 0·10$)	—
GROUP II				
Methylated ether	100	0·82	Very highly significant ($P < 0·001$)	—
GROUP III				
Dequalinium chloride 0·5%	100	1·47	Very highly significant ($P < 0·001$)	Significant ($P < 0·05$)
Alcohol 70%	100	1·56	Very highly significant ($P < 0·001$)	—
GROUP IV				
Iodine 1% in spirit 90%	100	2·02	Very highly significant ($P < 0·001$)	Not significant ($P > 0·10$)
Merthiolate (tincture) 1 in 1,000	100	2·18	Very highly significant ($P < 0·001$)	—
Cetrimide 0·5% and Hibitane 0·05%	100	2·1	Very highly significant ($P < 0·001$)	—
Roccal (benzalkonium) 1 in 40	100	2·22	Very highly significant ($P < 0·001$)	—
GROUP V				
Laurolinium 1%	100	3·16	Very highly significant ($P < 0·001$)	Highly significant ($P < 0·01$)
Laurolinium 5%	100	3·38	Very highly significant ($P < 0·001$)	—

P = statistical significance of decrease.

The agents in group IV were not significantly more active than the 70% alcohol of group III, but the two Laurolinium solutions in group V were much the most efficient of all the skin disinfecting agents tested.

It is appreciated that we have no definite proof that lubrols W and lecithin will inhibit Laurolinium in the concentrations used for the trials.

In view of these findings, 5% Laurolinium has now been adopted as the standard pre-injection skin disinfectant in all wards in the Portsmouth Group which are served by the Central Sterile Supply Department.

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A survey of staphylococci isolated in hospital practice

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SYNOPSIS In-patient and out-patient strains of coagulase-positive staphylococci were compared and showed a different phage group distribution. Group III predominated in the in-patient series and accounted for nearly all the antibiotic-resistant strains. The prevalence of phage type 80 in breast abscesses was noted. It was found that one in three coagulase-positive staphylococci isolated from hospital in-patients was sensitive to chloramphenicol and erythromycin only.

In 1948, Barber and Rozwadowska-Dowzenko drew attention to the increasing incidence of penicillin-resistant strains of *Staphylococcus aureus* isolated in hospital practice. Fairbrother (1956), examining strains from in-patients and out-patients, found penicillin sensitivity in 36% and 63% respectively. Hennessey and Miles (1958) reported a series of 248 strains of coagulase-positive staphylococci which were sent to them from East Africa, of which 40% were phage type 80; 37% were sensitive to penicillin.

MATERIALS AND METHOD

For a period of four months, from October 1958, to January 1959, swabs were taken from every abscess, infected lesion, or wound seen in the casualty department of this hospital.

All staphylococci isolated were tested for coagulase production by the tube method of Fisk (1940). Coagulase-positive strains were phage typed by the method of Williams and Rippon (1952). Antibiotic sensitivities were determined on agar plates using Evans Sentest tablets:

Penicillin	2·5 i.u./tablet
Streptomycin	80 μg./tablet
Tetracyclines	100 μg./tablet
Chloramphenicol	100 μg./tablet
Erythromycin	10 μg./tablet

In all 492 strains were examined.

For comparison, 500 strains of staphylococci from in-patients (sputa, boils, specimens of urine, wound, and nasal swabs) collected over the same period were similarly tested.

RESULTS

Table I shows the phage group distribution of all the casualty and hospital in-patient strains. The incidence of group I strains was the same in both groups; nevertheless, phage 80 staphylococci accounted for 10% of in-patient strains and only 4·5% of casualty strains.

TABLE I

GENERAL PHAGE GROUP DISTRIBUTION

Group	*I*		*II*	*III*	*I+III*	*I+II*	*IV*	*NT*
Phage	80	*Others*						
Casualty	23	177	110	97	27	0	5	53
Cases	4·5%	36·1%	22·5%	19·5%	5·5%	0%	1%	10·9%
In-patient	50	137	39	210	30	3	3	30
Cases	10·0%	27·1%	7·7%	42·0%	6·0%	0·6%	0·6%	6·0%

Group I = 29, 52, 52A, 79, 80

Group II = 3A, 3B, 3C, 55, 71

Group III = 6, 7, 42E, 47, 53, 54, 75, 78

Mixed groups, *i.e.*, with lysis to phages of two groups above are classified as such.

Group IV = 42D, 187 (and miscellaneous)

Table II shows the antibiotic sensitivities of the strains isolated according to source.

TABLE II

ANTIBIOTIC SENSITIVITY

	Casualty	*In-patient*
Number of strains isolated	492	500
No. penicillin sensitive	303 (61·5%)	117 (23·4%)
No. penicillin insensitive but sensitive to chloramphenicol, erythromycin, streptomycin and/or tetracyclines	173 (35·3%)	218 (43·6%)
No. sensitive to chloramphenicol and erythromycin only	16 (3·2%)	154 (30·8%)
No. erythromycin sensitive only	0	8 (1·6%)
Insensitive to all	0	3 (0·6%)
	492 (100%)	500 (100%)

Sixty-one per cent of the casualty strains were sensitive to penicillin whereas only 21% of strains from in-patients were sensitive. Thirty per cent of

in-patient strains were sensitive only to chloramphenicol and erythromycin whereas this was so in only 3% of casualty strains.

Group III staphylococci were responsible for most of the antibiotic-resistant organisms in both casualties and in-patients. They accounted for two-thirds of strains sensitive to chloramphenicol and erythromycin only (phage type 80 made up most of the remaining third), most of strains sensitive only to erythromycin, and for all those resistant to the five antibiotics tested.

In in-patients, 1·6% were sensitive to erythromycin only and 0·6% were insensitive to all five antibiotics.

TABLE III

PHAGE GROUPS OF CASUALTY STRAINS ACCORDING TO SITE

Group	*I*		*II*	*III*	*I+III*	*IV*	*NT*
Phage	80	*Others*					
Septic hand 249	9	104	70	35	8	2	21
Breast abscess 18	4	5	4	2	3	—	—
Axillary abscess 17	—	7	1	4	3	—	2
Perineal abscess 17	—	5	4	2	2	—	4

Septic hand included pulp space infections, paronychia, web space infections, etc., and yielded group I in 40% of cases, of which 3·5% were phage type 80.

Eighteen cases of breast abscess were included in the survey; of these, four had phage type 80 infection. All were sent a questionnaire to find if they had recently been delivered of a child, and if they had been admitted to a hospital or nursing home before the development of the abscess. Replies were received from 14 patients, but among the four who did not answer were two who had phage type 80 staphylococcal infection. Five of the 14, including both the patients with phage type 80 infection who replied, had recently been delivered in hospital.

TABLE IV

ANTIBIOTIC SENSITIVITIES OF PHAGE TYPE 80 STAPHYLOCOCCI

	Sensitive to: *Penicillin*	*Strepto-mycin*	*Tetra-cyclines*	*Chloram-phenicol*	*Erythro-mycin*
67 strains	1[1]	10	6	65	66

[1] Out of 68 strains; the penicillin-sensitive strain was not tested for sensitivity to the other antibiotics.

Although the antibiotic pattern of phage type 80 varies, the predominant one of these strains was sensitive only to chloramphenicol and erythromycin. No phage type 80 was insensitive to all the antibiotics tested.

We wish to thank Miss Ann Butcher for technical assistance, and Miss M. N. Ellis and Mrs. E. M. Bluck for typing this report.

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Staphylococci in septic fingers

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SYNOPSIS The phage patterns of 123 cultures of *Staphylococcus aureus* isolated from septic fingers were compared with 124 strains isolated from the noses of healthy volunteers. It was found that strains with certain phage patterns were comparatively numerous in septic fingers, and that the lesions caused by them tended to be exceptionally severe.

Some strains of *S. aureus* are very prone to cause primary skin infections. Notably are those with phage patterns 80 (Rountree and Freeman, 1955), 52A/79 (Alder, Gillespie, and Thompson, 1955) and some phage group II strains (Roodyn, 1954). Moreover, the infections caused by these strains (particularly phage type 80) are often exceptionally severe.

This investigation was undertaken to see whether a similar association existed among staphylococci causing septic fingers.

METHODS

The patients were seen in the Casualty Department. None had been given antibiotics. Those whose lesions had not localized and from which pus could not be examined bacteriologically, were excluded. The series of 138 patients was otherwise unselected.

The lesions were classified as to anatomical site, and their severity was assessed by the criteria shown in Table II.

Contact with hospitals during the preceding three months was noted, and included attendance at outpatient clinics and visits to relatives in the wards.

Pus taken at the time of incision was cultured aerobically on blood agar plates. Swabs were also taken from the patients' noses, and for comparison from the noses of 300 healthy volunteers resident in Bristol (238 blood donors and 62 patients with minor traumatic conditions attending the Casualty Department for the first time). None of the volunteers had attended hospitals in the 12 months before the investigation.

The bacteriological methods were those of Alder *et al.* (1955). Phage typing was carried out with the basic set of Colindale phages (Anderson and Williams, 1956).

RESULTS

The anatomical distribution of the lesions is shown in Table I. Cultures of *Staph. aureus* were obtained from 123 lesions. Of the remaining 15, three were sterile and 12 yielded a variety of organisms other than *Staph. aureus*.

All of the 24 dorsal carbuncles seen were pure staphylococcal infections, this being in accordance with the fact that a dorsal carbuncle on the finger is no different from carbuncles in other sites and is caused solely by *Staph. aureus*.

TABLE I

ANATOMICAL DISTRIBUTION OF THE 138 FINGER LESIONS

Causative Organism	*Total*	*Pulp Space Infections*	*Apical Compartment Abscesses*	*Dorsal Carbuncles*	*Subcutaneous Abscesses*	*Paronychia*
Staph aureus	123	19	11	24	23	46
Other organisms	12	1	2	0	5	4
No organisms isolated	3	0	0	0	1	2
Totals	138	20	13	24	29	52

SOURCES AND PHAGE PATTERNS OF THE STAPHYLOCOCCI

Table II shows the phage patterns of the staphylococci isolated from all sources. By comparison with the staphylococci from healthy volunteers, the lesions yielded proportionately more group I strains with the phage patterns 80 and 52A/79 (but not other group I strains). Group II strains which were insensitive to phage 3A, and the miscellaneous strains were also slightly more numerous in the lesions. The phage patterns of strains from patients' noses were similarly distributed to those from the lesions. It thus appeared that septic fingers were sometimes caused by staphylococci from the patients' carrier sites, as has been demonstrated with simple furunculosis (Roodyn, 1954; Tulloch, 1954; Tulloch, Alder, and Gillespie, 1960).

Phage group II strains which were lysed by phage 3A were not infrequently isolated from healthy noses

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TABLE II

PHAGE TYPES AND CLINICAL SEVERITY OF THE STAPHYLOCOCCI FROM SEPTIC FINGERS

Phage Patterns	*Healthy Noses*		*Patients' Noses*		*Septic Fingers*		*Criteria of Clinical Severity*		
	Total	*Penicillin-resistant*	*Total*	*Penicillin-resistant*	*Total*	*Penicillin-resistant*	*Slough Formation No. of Strains*	*Mean Duration of Disability (days)*	*Mean Time taken to Localize (days)*
PHAGE GROUP I									
Phage pattern 80	0	0	1	1	5	5	5 (100%)	27·5	4·2
Phage patterns 52A/79	15	4	19	6	34	15	24 (70%)	19·9	5·0
Other phage patterns	30	1	10	0	17	2	5 (29%)	18·8	4·8
PHAGE GROUP II									
Phage patterns with 3A	7	4	1	0	0	0	—	—	—
Phage patterns without 3A	24	2	18	3	30	3	15 (50%)	13·3	5·2
PHAGE GROUP III									
All patterns	13	5	8	6	10	6	2 (20%)	11·3	4·6
PHAGE GROUP IV	0	0	0	0	0	0	—	—	—
Miscellaneous	15	1	11	1	16	5	9 (56%)	14·1	4·6
Untypable	20	1	5	0	11	0	0	14·7	6·0
Totals	124	18 (14·5%)	73	17 (23%)	123	36 (29%)	60	—	—
Staph. aureus (included above) with group A streptococcus					15	—	0	13·0	2·3

but never from septic fingers. This finding was similar to that of Alder (1956) in strains from furunculosis.

There were only 10 septic finger strains in phage group III, and together they possessed nine different phage patterns; no strains reacted with phage 42D. There were 16 patterns in the miscellaneous group and 11 strains were untypable.

PHAGE TYPES AND CLINICAL SEVERITY The lesions caused by certain strains of staphylococci tended to be exceptionally severe. The more virulent strains were those which were found comparatively often in septic fingers as compared with healthy noses, *e.g.*, type 80 and 52A/79, phage group II strains insensitive to 3A, and the miscellaneous group (Table II). The association between sloughing and prolonged disability on the one hand and phage pattern on the other was particularly well marked with the phage group I strains. The severest lesions were caused by the type 80 staphylococci in accordance with what has been found with furunculosis (Rountree and Freeman, 1955; Gillespie and Alder, 1957), and patients experienced considerable malaise. It was also of interest that of the 46 paronychias in the series, the only one to form a slough was caused by the only type 80 strain isolated from a paronychia.

The presence of a combined infection with a group A streptococcus apparently shortened the clinical course, resulting in early localization (Table II).

RESISTANCE TO ANTIBIOTICS The penicillin resistance of the different phage types is shown in Table II. Thirty-six (29%) of the 123 strains from septic fingers were resistant to penicillin compared with only 18 (14·5%) of the 124 healthy nose strains. This difference could be accounted for by the greater number of type 80, 52A/79, and 'miscellaneous' strains in the septic finger group. All five type 80 staphylococci were resistant to penicillin, and three of these were resistant to other antibiotics as well.

Contact with hospitals was significantly associated with the presence of penicillin-resistant staphylococci in lesions. Of the 25 patients with hospital contacts, 16 (64%) were infected with penicillin-resistant staphylococci compared with 20 (20·4%) of the 98 other patients.

DISCUSSION

The assessment of virulence by the clinical severity of lesions presents difficulties, but in the present series certain strains, notably those with phage patterns 80 and 52A/79, together with some group II strains, were undoubtedly associated with unusually severe lesions.

The two most reliable measures of clinical severity were the duration of disability and the percentage of lesions which led to sloughing. The formation of a slough in a septic finger is therefore sometimes related to the properties of the causal staphylococcus and not only, as is sometimes taught, to faulty or delayed treatment. In the present series early sloughing was often a feature of the more severe lesions, particularly those caused by staphylococci with phage types 80

and 52A/79, and evidently had nothing to do with the treatment.

We wish to thank Mr. H. K. Bourns, surgeon in charge of the Casualty Department, Bristol Royal Infirmary, for his encouragement and permission to treat cases under his care; Dr. W. A. Gillespie for his help and criticism; and the Board of Governors of the United Bristol Hospitals for financial help to enable this work to be completed.

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Book Reviews

HOSPITAL INFECTION: CAUSES AND PREVENTION. R. E. O. Williams, R. Blowers, L. P. Garrod, and R. A. Shooter. (Pp. x + 307; 12 figures. 35*s*.) London: Lloyd-Luke, 1960.

In view of the increasing importance of staphylococcal infections in hospitals, it is not surprising that the accent in this book is on those. Nevertheless, in spite of this preponderance, the general principles of dealing with hospital infection are well exemplified in all their aspects. The book is divided into parts dealing with infections occurring in all types of wards, where, for example, the infectious diseases are separately dealt with, and nursing precautions for all of them are clearly described. Very short passages on such subjects as venereal disease and diphtheria could well have been omitted but help to make the book complete. The best parts are those dealing with the administrative control of hospital infection, which show clearly the duties which would be expected of a control of infectious disease officer, together with the local public health authorities. In fact the book is clearly written with the idea of hospital officers, through their bacteriologists, calling in the public health authorities and the public health laboratory services, who would act as epidemiological officers in tracing infections. This is in fact a little idealistic inasmuch as most hospital bacteriologists could, or should, be able to deal with their local infections with their available resources, using the public health laboratories as reference laboratories. The book is authoritative and up to date on all questions dealing with resistant staphylococcal infections in hospitals. It clearly shows where the difficulties have arisen, and makes great use of the present grave situation as regards staphylococci to suggest better use of the known and well-tried aseptic techniques, which still should have been in use in operating theatres and wards. These chapters trace sources of infection in theatres with wrongly designed ventilation, in wards with irresponsible techniques, and in fact lay down a series of guides to hospital officers for steps which should be taken in outbreaks of single cases or epidemics of staphylococcal infections. It is also clear that the authors do not place much reliance on the paraphernalia that go with the usual type of hospital infection control. The reader, be he pathologist or clinician, can find representative examples of infections or epidemics, and the authors are each quoted throughout the book in their several experiences in ward and theatre infection. In the last chapters there is a wealth of information on technical methods of sterilization; the autoclave is dealt with in a most satisfactory way, but this section would certainly not be understood by the majority of the nurses or porters who have to deal with the instrument, as it is written more for the scientific worker, and underlines the fact that the person in charge of the autoclave has to be knowledgeable about bacteriology and the physics of the instrument. A very short section deals with sterilization by dry heat, describing the faults of the ordinary hot air oven, and all too brief paragraphs mention the infrared tunnel and the future sterilization by gamma-radiation. The chapter on practical sterilization and disinfection is excellent. This is the sort of chapter which can be read by any nurse who really needs to know how to sterilize individual things like sputum mugs, urinals, or even the skin of surgical attendants! On the whole, therefore, this book can be strongly recommended to all hospital infection committees and their members, who have at this moment to face the troublesome, persistent, and recurring epidemics of staphylococcal infections, and can certainly learn from this book how to deal with outbreaks in their own hospitals.

A. GORDON SIGNY

RECENT ADVANCES IN CLINICAL PATHOLOGY, Series III. By various authors. General Editor: S. C. Dyke. (Pp. xii + 425; 151 figures. 50*s*.). London: J. & A. Churchill. 1960.

The publication of this volume was delayed for a number of reasons which the senior editor explains in his preface. In fact nine years have elapsed since the last *Recent Advances in Clinical Pathology* appeared and these years have seen a tremendous development, both qualitative and quantitative, in the subject. In fact, the four main divisions in pathology have shown signs of growing apart in this period, and their practitioners have become more and more specialized. Each year too sees the incorporation into the accepted routine examinations done in a hospital laboratory of many new investigations, which were a short time before regarded as purely research tools. The choice of subjects therefore for a book purporting to be 'recent' must be a difficult decision for the editor. In the past he has felt that discussions of accepted procedures in investigational medicine were all that was required, but in this number it is clear that the aim has been to acquaint and bring up to date the clinical pathologist with the urgent newer techniques which he will be asked to provide by his clinical colleagues. Thus he can learn how to control staphylococcal infections in hospitals, to diagnose toxoplasmosis, and to talk intelligently with the virologist. In the chemical pathology section the aim has been to describe the newer work on four separate metabolic disorders and these chapters are excellent, although the terminology is occasionally very difficult because of the numerous abbreviations which have crept into the nomenclature. A more technical chapter on paper electrophoresis is relieved by an all too brief survey of the clinical applications.

The histopathological section sets a very high standard in photomicrography, of which the photographs of glomeruli obtained by needle biopsy are quite outstanding. There is an excellent description of the technique of this biopsy and no doubt many pathologists will be tempted to attempt such biopsies hoping to obtain as good diagnostic results. The chapter on fungous

infections also deserves special mention, especially for the excellence of the illustrations.

It is strange to find a chapter on rheumatism and connective tissue diseases amongst the more orthodox haematological subjects, but this is the penalty of having sections in such a volume. In fact the haemoglobinopathies could, for example, well be called chemical pathology, the fungal infections could be included in the bacteriological section, and it appears that even this book may well encourage those who regard the four main branches of pathology as four quite divorced disciplines. Yet this is obviously not the intention of the General Editor.

The General Editor points out that this new series does not mean at all that the earlier editions are now out of date and superseded by this edition. On the contrary, entirely new subjects have deliberately been chosen for inclusion. It becomes therefore even more imperative that there should not be a delay of anything like nine years before the fourth series appears. In fact the hospital pathologist will be demanding a regular flow of such excellent surveys to enable him to carry out the functions so well described by the General Editor in his preface.

A. GORDON SIGNY

THE BRITISH MEDICAL BULLETIN, Vol. 16, No. 3. 'Insulin'. London: British Council. 1960.

The current number of the *British Medical Bulletin*, dealing with insulin, is no exception to the extremely high standard of the various special numbers.

After introductory historical surveys by Young and by Best, an excellent review by Sanger of his brilliant work on the chemistry and structure of insulin is followed by an account of oxytocin, vasopressin, corticotrophin and melanotrophin, polypeptide pituitary hormones, the structures of which have been elucidated by methods applied by Sanger. Methods of insulin assay are described by Stewart: the extension of these to the investigation of plasma insulin and its antibodies and other antagonists has been critically discussed by Randle and Taylor, by Valance-Owen, and by P. H. Wright. If anything, in this work there is perhaps an overemphasis on and preoccupation with methods employing the rat diaphragm.

The critical reviews of the action of insulin on carbohydrate metabolism and on fatty acid metabolism by Fisher and by Folley and Greenbaum respectively are the most stimulating of all the contributions. Fisher discusses critically the hexokinase hypothesis of the action of insulin and the possible role of insulin in increasing the permeability of cells to sugar, a possibility later considered by Randle and Young. Fisher tritely remarks that 'if it is true that the major action of insulin in relation to carbohydrate metabolism is to regulate access of glucose to peripheral cells, then in the biochemical sense it may have no action at all on carbohydrate metabolism'.

Folley and Greenbaum make it clear that there are two defects in insulin deficiency, first in the conversion of extracellular glucose to intracellular glucose-6-phosphate and a second defect at the level of conversion of acetyl coenzyme A to the 4-carbon stage of the synthesis of fatty acids. Considering work on the liver, mammary gland, and adipose tissue, they discuss the possibility that the primary action of insulin might be to facilitate glucose oxidation and so provide TPNH via the pentose phosphate pathway and thus secondarily stimulate fatty acid synthesis in contrast to primarily stimulating the synthesis of fatty acids and by oxidizing TPNH to TPN provide a stimulus to carbohydrate metabolism.

Korner and Manchester have discussed the relationship of insulin to protein metabolism and describe the work in this field leading up to their own observations that insulin *in vivo* can affect the ability of microsomes to assemble activated amino-acids into polypeptide chains. On the other hand, Randle and Young consider that insulin is most likely to play an intracellular role separate from its effect on the entry of substances into the cell, but confess that it is not known at present whether or not the many effects of insulin are likely to be explicable in terms of a single action.

The four clinically biased papers are excellent and include an account of the relationship of endocrine disorders and insulin action by Russell Fraser, and a highly critical account of the clinical applications of insulin by Oakley who confesses that the long-acting insulins have not fulfilled all that had originally been hoped; he admits to the impossibility of reproducing blood sugar control comparable with that of normal subjects even with frequent doses of insulin. The relation between the molecular structure and hypoglycaemic activity has been considered by Mahler. Indeed, the development of the oral hypoglycaemic agents has been highly empirical and it is still impossible to determine the chemical or physical feature responsible for their activity. Nabarro provides a valuable account of the clinical effectiveness of the various insulin substitutes but states that it is too early 'to attempt any long-term assessment of the place of any sulphonamide derivatives in the treatment of diabetes mellitus'.

It is becoming a commonplace that acquisition of knowledge passes first through a phase when all seems straightforward and easily understood, only to be followed by a phase of great complexity. Our knowledge of the functions and mechanism of the action of insulin is no exception. When insulin was first isolated 40 years ago, all seemed simple and it would then have been difficult to foresee that even now we would still be ignorant of so much of this subject. This special number of the *British Medical Bulletin* provides a synthesis of present-day knowledge which will be invaluable for all concerned with this hormone, whether research workers or clinicians essentially preoccupied with its therapeutic use.

CHARLES H. GRAY

DISEASE AND THE ADVANCEMENT OF BASIC SCIENCE. Edited by Henry K. Beecher. (Pp. xi + 416; illustrated 100*s*.) Harvard University Press (London: Oxford University Press). 1960.

This book, based on the Lowell lectures of 1958, is concerned with the stimulus given by medical studies to investigations in the basic sciences. The first chapter is by Linus Pauling and the last by René Dubos and the long list of contributors is a distinguished one. The range of

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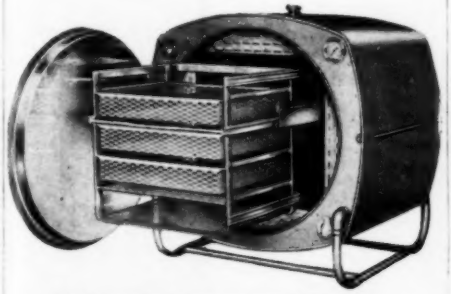

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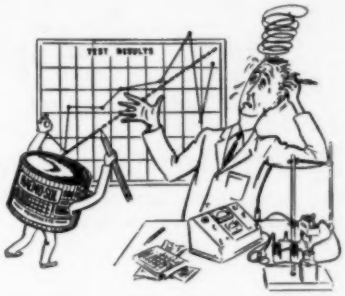

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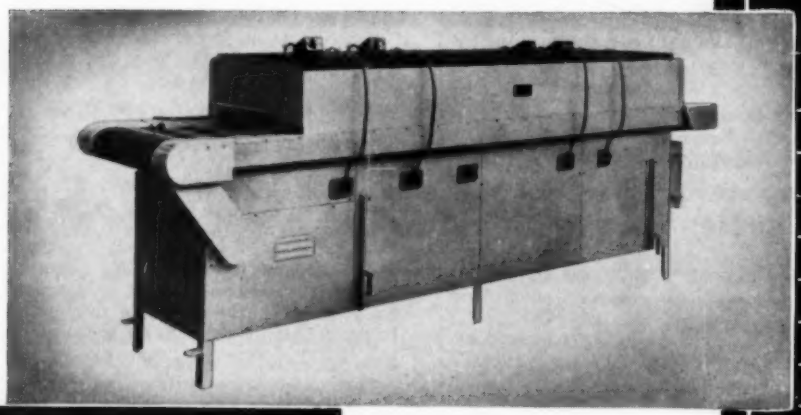

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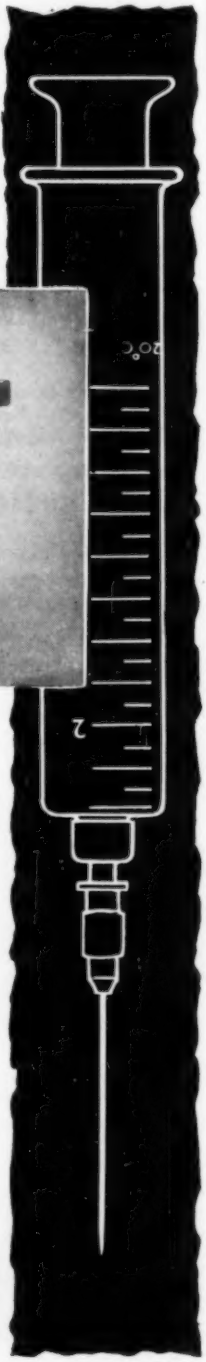

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topics is wide indeed, with Cori writing on carbohydrate metabolism, Enders on viruses, Castle on physiological advances stimulated by studies on anaemia, Bauer on rheumatic diseases, Aub on cancer and normal cell growth, and many others. The theme of the series of lectures is brilliantly illustrated and it is good to be reminded that medicine is not always the beneficiary in the relationship between itself and the basic sciences. The moral which emerges, however, is that the relation is essentially one of mutual dependence and, as Paul Weiss says, 'The meaning of setting the two apart, as if they were opposites or even separable, escapes me. Clinical investigation and biological research are integral parts of the same endeavour'. In the last chapter, Dubos, in a sombre contribution, points out that medical Utopias, no less than political Utopias, are likely to prove illusory and he warns that unless medical scientists are prepared to take the long view they 'may come to know the anguish that atomic scientists experienced as they watched the tragic effects of their scientific triumphs'. This is a book for every pathologist with a live interest in the relation between his work and the whole body of science. Most of it is easy to read, all of it is stimulating, and many of the contributions have the merit of posing more questions than they answer.

F. HAMPSON

BIOCHEMICAL VALUES IN CLINICAL MEDICINE: THE RESULTS FOLLOWING PATHOLOGICAL OR PHYSIOLOGICAL CHANGE. Robert Duncan Eastham. (Pp. iv + 144. 15*s*.) Bristol: John Wright. 1960.

The average medical registrar will find this book attractive because it gives him, in summarized form, the means of interpreting chemical pathological results. But it is impossible in a book of this size to indicate the criteria of utility of any investigation; a critical approach to laboratory results is essential if the patient is to have proper care and therefore this book is unlikely to help the patient. There are one or two factual errors, *e.g.*, the alkaline phosphatase tends to rise with advancing years, not to fall. A number of investigations have been included, such as the determination of β-glucuronidase in urine and the serum diphenylamine reaction, which are not in common use and would be better omitted. The limitations of other investigations might have been brought out more clearly: for example, the congo red test is not usually helpful, Bence-Jones protein is commonly absent from the urine in myelomatosis, and the hippuric acid synthesis test yields unsatisfactory results in the presence of a peptic ulcer. There are always statements with which one must disagree, and amongst these must be included the statement that the upper limit of the serum cholesterol level in adults is 250 mg./100 ml. (it is higher than this and increases with age), and the failure to make clear the significant relationship between the average daily protein intake and the blood urea clearance. The statement that estimation of radioactive iodine uptake by the thyroid gland is more accurate than estimation of the B.M.R. may be true if iodine has not been exhibited recently, but the investigation is one to be avoided in the youthful; qualifications such as these must not be omitted.

ARTHUR JORDAN

The Association of Clinical Pathologists

SUMMARIES of two papers presented at the meeting of the Association of Medical Pathologists and the Association of Clinical Biologists on 29 October, 1960 at the Royal College of Surgeons, London, follow. They were omitted from the full account of the meeting published in the November issue of the Journal.

MINIMAL CRITERIA FOR THE DIAGNOSIS OF MEGALOBLASTIC ANAEMIA OF PREGNANCY

I. RANNIE and H. MCTAGGART said that during a 21-month period 45 cases of megaloblastic anaemia of pregnancy were diagnosed in a series of 3,741 women delivered in hospital.

Specimens of venous blood were collected with Sequestrin as anticoagulant and in most cases were not received in the laboratory until the following day, having been stored overnight at room temperature or 4°C. and then transported by van.

When the specimen was received the haemoglobin was estimated. If this was 75% (11·1 g.%) or below, or if the case was already being followed, the reticulocytes and packed cell volume were estimated and a buffy coat film was made.

The packed cell volume was estimated using a Hawksley micro-haematocrit centrifuge, after which the capillary tube containing the blood was cut and the buffy coat and some of the underlying red cells were used to make a smear which was stained with Leishman's stain.

The diagnosis of megaloblastic anaemia was not made until megaloblasts appeared in the peripheral blood but the diagnosis can in fact be established before this time by finding macrocytes, polylobing of polymorphonuclear leucocytes and normoblasts in which the degree of haemoglobinization is in advance of nuclear maturation.

Details of four of the cases were given, with special reference to time of diagnosis, the buffy coat film, and the appearance of megaloblasts in the blood following intravenous or intramuscular iron therapy.

Accurate haemoglobin estimations are an important part of antenatal care. Any haemoglobin found to be below 75% (11·1 g.%) should be followed by the examination of buffy coat films so that folic acid deficiency anaemia of pregnancy can be diagnosed and treated as early as possible.

METABOLIC CHANGES FOLLOWING SEVERE TRAUMA

I. G. GRABER said that mortality from severe skeletal trauma has been reduced from 40% to 1·4% in the last decade largely due to adequate blood transfusion, but

in injuries involving the thorax, head, abdomen, and skin, the mortality remains high.

To define the problem, two groups of adequately transfused severe trauma cases were studied: 1, 11 cases of skeletal trauma; 2, four thoracic, four head, four abdominal, and 13 thermal injuries.

Replacement therapy was controlled by frequent blood volume estimations.

Creatinine clearances, tubular handling of electrolytes, blood pressure, and pulse rates were measured at 15-minute intervals initially, one or two hourly for at least 100 hours and 12-hourly for three weeks. Serial cardiac output data were available in six patients.

In skeletal trauma, large blood transfusions caused large fluctuations in each function studied. These varied in amplitude and period with time after injury and for different functions. Values for Na/K ratio and percentage filtered water and sodium excreted varied about a near normal mean.

Those with thoracic injuries showed persistently low cardiac output, Na/K ratios, and water and sodium retention with a normal blood volume, presumably due to disturbances of right to left heart blood flow and baroreceptor function. A similar pattern shown in abdominal injuries coincided with the presence of ileus. Head injuries with minimal blood transfusion showed large oscillations in blood pressure, pulse rate, and renal function. Extensive burns resulted in larger fluctuations of renal function and more damping of oscillations than the other groups, depending upon the clinical course. The effect of drugs, including anaesthesia, upon these patterns varied with the metabolic state.

It is known to engineers that when regulating mechanisms dependent upon feed-back control are subjected to disturbances, they may pass through a period of oscillation before returning to a steady state. Under certain conditions, certain forms of interference may cause extreme fluctuations and result in irreversible damage.

Blood transfusion, anaesthesia, and physiologically active drugs represent such disturbances to inherent homeostatic mechanisms maintaining the internal environment after trauma, particularly when organs concerned with regulation of the internal environment are injured (Graber, 1959).

Data were presented from the literature to indicate that similar fluctuations occur after ureterocolic anastomosis, adrenalectomy, and heart operations.

The interrelationships between the functions studied were not linear, but if they were known quantitatively and in detail, the behaviour of the system might be predicted and therapy designed to avoid extreme fluctuations. For the present this is not possible; far more study is needed. The results indicated the futility of isolated biochemical estimations and clinical observations as a guide to therapy.

Graber, I. G. (1959). *Modern Trends in Accident Surgery and Medicine*, Chapter III. Butterworth, London.

Notices

NO. 9 FREEZE-DRIED SERUM

We have received a notice from C. Davis Keeler Ltd. that the new batch No. 9 of freeze-dried serum prepared by the Glaxo Laboratories and analysed by the A.C.P./A.C.B. Committee is now available for distribution. The cost is 4*s.* 6*d.* per vial and the analysis covers the same constituents as before.

FOURTH INTERNATIONAL CONGRESS ON ANGIOLOGY

A preliminary programme has been received from the organizers of the Fourth International Congress on Angiology. The subjects will cover the metabolism of the vascular wall. The Congress will take place in Prague from 4 to 9 September, 1961.

INTERNATIONAL CONGRESS OF NEUROLOGY

The Seventh International Congress of Neurology will be held in Rome from 10 to 15 September 1961 under the auspices of the World Neurologic Federation and of the National Institute for Nervous Diseases and Blindness of Bethesda.

Full details are available from the President of the Congress, Prof. Mario Gozzano, or from the Secretary General, Dr. Giovanni Alemà, Viale Università 30, Rome.

J. clin. Path. (1961), **14**, 103

Mortuary design and hazards[1]

A committee was set up in November 1959 to investigate the conditions under which necropsy work is carried out, and to report to the Council on any desirable improvements in these conditions.

The committee has circulated questionnaires to pathologists in a number of different areas of the country, and has collected information regarding existing facilities for necropsy work in general hospitals, teaching hospitals, and public mortuaries. Views have also been sought on the need for improving mortuary facilities, and on desirable standards for these three types of mortuary unit. Members of the committee have discussed the subject with numerous pathologists, with hospital architects, and with other interested parties, including coroners and medical officers of health. The present memorandum presents the committee's conclusions and recommendations.

RESULT OF THE SURVEY

The committee was impressed by the extremely varied standards existing in different hospital departments and public mortuaries throughout the country (Figs. 1 and 2). Some mortuary units are spacious and well-designed and are well-staffed and equipped with every facility for efficient work. A large number, however, are cramped and awkward, with few facilities, and the committee feels that there is a general need for improvement. As new hospitals are built, this improvement is gradually being effected by the provision of modern and well-designed mortuary units, but there is urgent and very considerable scope for the improvement of mortuary units in existing hospitals: the same is true with regard to public mortuaries.

The committee feels that information and general guidance should be available for those pathologists who have the opportunity either of designing new units or of improving old ones: it hopes that such information will stimulate many pathologists to look afresh at their own mortuary facilities and see what can be done to improve them.

Many pathologists have expressed dissatisfaction with staffing arrangements in mortuaries, and the committee has some comments to make on this subject.

The question of the joint use of mortuaries by hospital and coroners' pathologists is also considered.

DESIGN AND EQUIPMENT OF MORTUARIES AND POST-MORTEM ROOMS

Although general considerations of design and equipment apply equally to various types of mortuary unit, both hospital and public, there are differences of emphasis and detail, and we have thought it best to set out our views under headings relating to the large general hospital, the smaller hospital, the teaching hospital, and the public mortuary. It will be clear, however, that much of what we have to say regarding hospital units applies to public mortuaries.

LARGE GENERAL HOSPITAL In designing a new hospital it is desirable to incorporate the mortuary unit, including the post-mortem room, in the main structure. Access to the unit is of great importance, and it should be sited so that there is ready and separate access by the various groups of people concerned (pathologists and other medical staff, staff transferring bodies from wards to mortuary, relatives coming to view bodies, undertakers, etc.). Sometimes there may be conflict between these different requirements, but in a modern hospital block good access is often most readily achieved by siting the mortuary unit in the basement. There can be separate routes of entry within the hospital for bodies, visiting relatives, and medical staff. An external undertakers' entrance, screened from wards and outside roads, can be provided.

In existing hospitals, the mortuary unit is usually a separate building, and this may sometimes be an advantage if it is to be used jointly by the hospital and local authority (see page 107). Siting and access are still important matters. A separate mortuary unit should have pleasant and dignified surroundings, and should not—as is so often the case—be consigned forever to some remote, inaccessible, and untidy corner of the hospital grounds.

In addition to a general mortuary area equipped with adequate refrigerated body storage, and a suitably sized and equipped post-mortem room, the mortuary unit should include a room for the mortuary attendant, facilities for viewing bodies, and a waitingroom and W.C. for visiting relatives.

[1]Report to the Council of the Association of Clinical Pathologists of the Committee on Mortuary Design and Hazards (F. E. Camps, chairman, G. Stewart Smith, A. Dick, and H. H. Sissons, secretary).

FIG. 1

The mortuary area must be large enough to provide space for undertakers; adequate storage space is important. Facilities for washing and gowning should be provided outside the actual post-mortem room, and shower rooms and W.C.s should be available both for pathologists and post-mortem room attendants. If the unit is at a distance from the pathology department, a doctors' room should be provided where reports can be written or dictated. Some pathologists would like such a room, or the pathology department itself, to be linked to the post-mortem room by a two-way inter-communication system, so that reports can be dictated to a secretary or recorded on tape during the course of a necropsy.

An actual 'chapel' for the viewing of bodies is regarded as unnecessary. A simple but quite adequate scheme can consist of a viewing room, separated from part of the mortuary area by a curtained window, the glass of which is tilted from the vertical to avoid reflections. The body to be viewed can be laid out in the mortuary, completely separated from viewers by the window: it can be isolated from the rest of the mortuary area by sliding curtains hanging from the ceiling. A separate room to house a body to be viewed is even better: again, a glass window should effect complete separation between the body and the viewing relatives.

There is abundant scope for individual planning in the arrangement of the rooms making up the mortuary unit, and this should always be considered with the hospital architect. Thought should be given to the different routes by which bodies and various types of personnel move into and out of the unit: these should overlap as little as possible. On no account should there be a common entrance or exit for bodies and visiting relatives. It is most undesirable that relatives should have access by any route to the mortuary area itself or to the post-mortem room. The mortuary attendant's room should be sited so that he is available to visiting relatives, as well as having ready access to the mortuary area and the post-mortem room. Con-

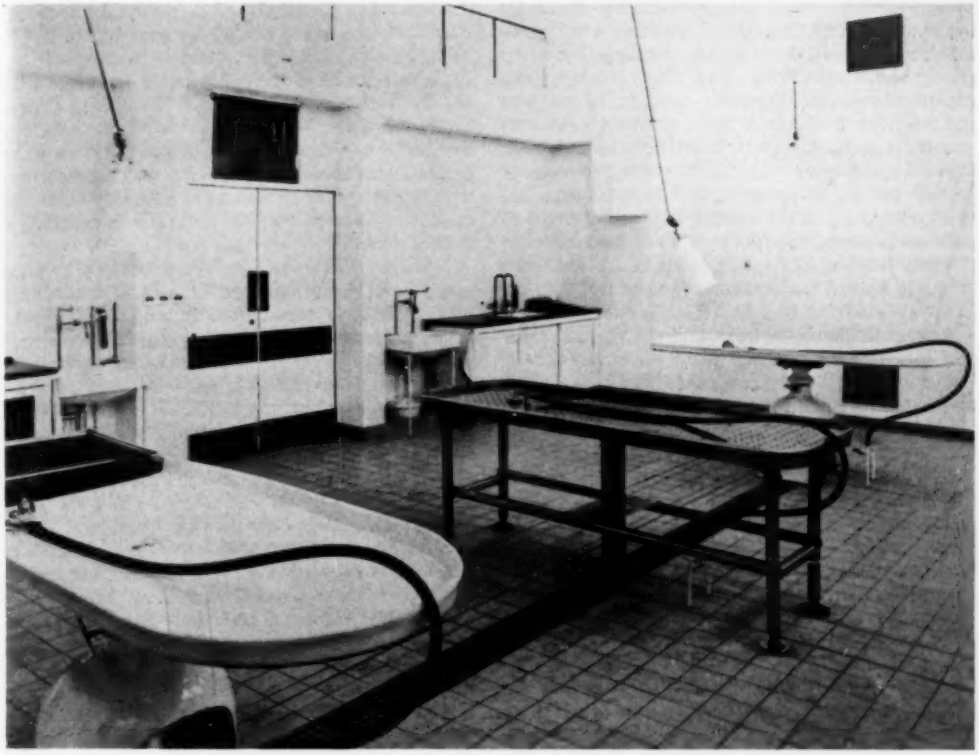

FIG. 2

sultation with the hospital architect and reference to such works as Rosenfeld's 'Hospitals: Integrated Design' (2nd edition, Reinhold, New York, 1951), and 'Studies in the Function and Design of Hospitals', a report of an investigation sponsored by the Nuffield Provincial Hospitals Trust and the University of Bristol (Oxford University Press, 1955), are suggested for those concerned with the design of a new unit.

Body storage Refrigerated body storage is of course essential. It should be provided on a generous scale, particularly when a mortuary is to be used for both hospital and outside coroners' cases. A mortuary where 500 necropsies a year are carried out should have storage space for at least 18 bodies: storage space for 24 to 30 is usually adequate for 1,000 necropsies a year.

Post-mortem room Adequate space is essential: the committee feels that a poor standard of work is more often due to cramped conditions than to any other single factor. A post-mortem room dealing with 500 necropsies a year should, if possible, have three post-mortem tables, and its floor area should be not less than 500 sq. ft. A post-mortem room with four tables should have a floor area of approximately 800 sq. ft. If air conditioning is not available, the post-mortem room should be of generous height (12 to 15 ft.) and there should be adequate ventilation. In any case, the whole unit should be fly proof.

Air conditioning is highly desirable, and should certainly be considered when any new unit is planned. It should provide a high rate of change of air, it should not involve recirculation, and it is important that air extracted from the mortuary is not delivered to other parts of the hospital, even indirectly. In this connexion, the mortuary unit should not be directly in communication with a lift shaft or with any other shaft which has openings on other floors.

Lighting is extremely important. Good daylight is satisfactory, but the situation of the post-mortem room may make it impossible to provide this. If the main sources of illumination is by artificial lighting this needs careful planning. The general level

of illumination should be approximately 30 lumens per sq. ft., with additional lighting over post-mortem tables and dissecting benches. Provision should be made for some form of moveable lighting to examine cavities. The pros and cons of tungsten lighting and fluorescent lighting (natural colour) should be considered: it is difficult to recognize important colour changes (such as that concerned in carbon monoxide poisoning) with some types of artificial lighting. Roof lighting is often a feature of older mortuaries, but roof lights can interfere with the maintenance of an even temperature and need shielding against brilliant sunshine: we feel they are better avoided.

A smooth finish to walls and floors makes for easy maintenance of a high standard of cleanliness. Terrazzo finish has been used in recent years but may crack: modern materials such as polyvinyl chloride (P.V.C.) sheeting can be used to give an impermeable and easily washable surface for both walls and floor with rounded surfaces in all corners. Conduits, pipes, etc. can be located behind flush removable panels. Acoustically absorbent material should be used for the ceiling. Cupboards and other furniture should be plinth-mounted to facilitate washing down.

Plenty of well-illuminated bench space (say 20 ft.) should be provided for dissection, weighing of organs, etc., away from the post-mortem tables. It should include several large sink units, fitted with hot and cold water, and the bench top should be of impermeable material, preferably stainless steel. The surface actually used for dissection should be of easily cleaned material such as hard wood. Specimens which are to be preserved for museum mounting should not come in contact with water: the provision of running saline allows the washing of such specimens prior to their fixation. There should be adequate cupboard space (preferably built-in) for instruments, specimen jars, etc. Storage space for clean gowns, boots, aprons, gloves, etc. should be located outside the post-mortem room. A cleaners' cupboard for brooms and other cleaning materials should be provided in the mortuary area: there should also be adequate storage space for clothing of the deceased. Storage space (usually shelving) for fixed specimens should be provided near the post-mortem room, but a mortuary store room is the best way to overcome this difficulty.

In the post-mortem room, balances, blackboard, wall-mounted *x*-ray viewing boxes, and electric saw (reciprocating type) should all be standard equipment: a bandsaw and a portable *x*-ray unit should be available in a separate room, and the post-mortem attendant should be properly trained in the safe use of the latter. While porcelain post-mortem tables are commonly used, stainless steel tables do not chip and can more readily be kept clean. Tables should be provided with running water and with dissecting tables if these are used. Drainage from the table should be by completely enclosed pipes under the floor. If open floor channels cannot be avoided they should be covered by rust-proof metal grids. There should be provision for sterilization of instruments from potentially infective cases; an electrically heated instrument boiler is suitable, if properly used.

Hygiene With adequate space and proper planning, the post-mortem room should be as clean and efficient as an operating theatre, and the likelihood of bacterial contamination of staff during a necropsy should be minimal. As in an operating theatre, the provision of smooth washable surfaces and the avoidance of wall-mounted pipes and other obstructions can eliminate the accumulation of potentially infective dust and dirt. Strong emphasis should always be placed on hygiene in necropsy work. A good rule is that no one should enter the post-mortem room without donning gown and boots, and these must be discarded on leaving. A telephone can be provided within the post-mortem room which operates without being touched by hand. Other comparable measures can easily be instituted in a well-planned unit, but it is important to be reminded that in existing mortuary units a good standard of hygiene is often made impossible by bad arrangement of rooms and by cramped space.

The precautions that should be taken to prevent the dissemination of tubercle bacilli in the post-mortem room have been summarized in a report to the Public Health Laboratory Service[1]. The report stresses general cleanliness, training of post-mortem attendants, and disinfection of instruments, dissecting surfaces, protective clothing, etc., after necropsies on tuberculous subjects.

SMALLER HOSPITALS The general emphasis on access and on good facilities and cleanliness, noted for the larger unit should still apply; because a hospital is small, there is no reason that the standards should be lower. A post-mortem room with one table (adequate for about 100 necropsies per year) should have a floor area of 300 sq. ft., and should have the same standards of finish and equipment suggested for the larger unit. There should be facilities for washing and gowning outside the post-mortem room. The unit should include refrigerated body storage. A hospital carrying out 100 necropsies per year needs space for six bodies, and three is the minimum

[1] 'Precautions against tuberculous infection in the diagnostic laboratory', 1958. *Bulletin of Ministry of Health and Public Laboratory Service*, 17 January 1958.

number for any unit where necropsies are performed. However small the unit, there should always be facilities for viewing bodies, together with a waiting-room and W.C. for visiting relatives. The separation of access for doctors, visiting relatives, and for bodies and undertakers is as important in the small unit as in the large one.

TEACHING HOSPITAL The mortuary unit should be similar to that in a large general hospital. Because of the greater number of people likely to be present at necropsies, even more space is required in the post-mortem room itself. For the same reason, considerations of hygiene are of outstanding importance. Students and other onlookers should not enter the actual post-mortem area unless they are gowned and taking part in the examination: a tiered gallery with separate entrance should be provided for them, and this should be separated from the dissecting bench by a low glass screen to avoid the dispersion of potentially infective material during dissection of organs. (The same separation of an 'observer area' should be applicable, on a smaller scale, in non-teaching hospitals. It can be of great value when numerous visitors enter the post-mortem room and when the individuals concerned are reluctant to put on gowns and boots.)

A room for the fixation and storage of post-mortem specimens before their dissection and histological study is always needed in a teaching unit, and is an asset in any department. It should be adequately ventilated to avoid accumulation of formalin vapour. When it is desirable to store unfixed material, either for teaching or research, the provision of a cold room should also be considered.

PUBLIC MORTUARY These should be designed and equipped to at least the same standard and with the same care as hospital mortuaries. A working party has reported on standards for space and general arrangement for the Ministry of Housing and Local Government.[1] In this publication stress is laid upon the siting of doors for the entrance of relatives and the entry and removal of bodies. The proper siting of the mortuary itself is important. A large amount of body storage is needed in a public mortuary (provision for at least 30 bodies is required in a unit with 1,000 necropsies per year), and there should be proper facilities for viewing of bodies, *x*-ray examination, preservation of material, and storage for clothing, together with waitingroom and W.C. for relatives. The coroner's officer should be able to interview relatives, and the needs of undertakers and police should be borne in mind. Adequate accommodation with showers and W.C.s should be available for pathologists and mortuary staff.

Special provision needs to be made for dealing with decomposed and malodorous bodies. A separate refrigerator compartment should be reserved for this type of case, and a special metal or metal-lined casket provided for body storage. Special fan-extraction of air should be provided over one post-mortem table where such bodies can be examined. It is desirable to provide a separate room for this type of case, where possible, and such a room is also useful for detailed and lengthy examinations.

STATUS AND SALARIES OF MORTUARY ATTENDANTS

The work of the permanent mortuary staff, whether in hospitals or in public mortuaries, is responsible and exacting. There has been great difficulty in recruiting suitable people for this work, and our survey leaves us in no doubt regarding the dissatisfaction of many pathologists with the status and salary scales attached to the posts concerned. In many hospitals mortuary attendants are graded as belonging to the unskilled 'porter' class, while in public mortuaries the status and salary are little better. The committee feels strongly that this is wrong, and would like to see considerable improvement in the status of mortuary attendants, and in the salary scales which apply to them. We suggest that their position in hospitals should be comparable with that of laboratory technical staff. Similar changes are necessary in public mortuaries. The superintendent of a large public mortuary, to take one example, should be comparable in status and salary with a public health inspector. Such changes, we feel, would encourage the recruitment of suitable staff, and so increase the efficiency of mortuary work. It would also make the individuals concerned less dependent on the perquisites of their office—sums that come from a number of sources, not least the pocket of the pathologist whose job it is to carry out examinations in their particular post-mortem room.

JOINT USE OF MORTUARIES BY HOSPITAL AND CORONERS' PATHOLOGISTS

We have been impressed by what we regard as unnecessary duplication of mortuary facilities in some areas, and we feel that, in certain circumstances, there is scope for the joint use of mortuaries for hospital and coroners' necropsies. In big cities, the large number of coroners' necropsies makes it inevitable that these are carried out in special units and this is, in fact, desirable. But in smaller towns

[1] 'The Planning of Mortuaries and Post Mortem Accommodation'. (1956) H.M.S.O.

and in country areas it often appears uneconomic and inefficient to provide dual facilities for the same type of work. In some areas, it is already the practice for the hospital mortuary to be used for all coroners' necropsies from the surrounding district; in many others, conditions would appear to justify the adoption of similar units which could be designed to combine the functions of hospital and public mortuaries. Arrangements of this type need, of course, to be discussed and agreed by the coroner, the hospital authorities, and the local authority. Various administrative arrangements are in force, but the basic plan is for the local authority to pay for the use of what is, in effect, the hospital mortuary unit. This can be done either by an annual contribution or by payment for each body admitted. Apart from the added efficiency, such a scheme can be financially attractive, as the local authority is under a statutory obligation to provide, if requested, a public mortuary, and to do this properly as a separate scheme can be very expensive. The size of a shared unit, its architectural layout, and the designation of the facilities required in it, all need careful planning by the parties concerned.

It appears to us, then, that hospitals and local authorities might find it mutually advantageous to collaborate more regarding the joint use of mortuaries, and we feel that this topic should be the subject of their joint discussion, following the approval of the coroner, whenever the provision of a new mortuary unit—either hospital or public—is considered.

J. clin. Path. (1961), **14**, 109

Sclerosing osteogenic sarcomatosis

LIONEL WOLMAN

From the Department of Neuropathology, Royal Infirmary, Sheffield

SYNOPSIS A case is reported in which the amputation of a leg for a sclerosing type of osteogenic sarcoma was followed five years later by a metastasis of similar appearance in the skull. This was excised but subsequent necropsy revealed multiple bony tumours in the lungs.

The rarity of multiple osteogenic sarcoma is recalled as shown by a review of the literature. Although some are thought to be of multicentric origin owing to the short history, an early haematogenous spread cannot be excluded and this latter process had certainly occurred in the present case.

The characteristic radiological appearances in the skull are stressed as being diagnostic. The differential points in the histological diagnosis of the condition, especially in the invaded part of the calvarium, are described and should help to exclude a meningioma or other bone tumours.

Osteogenic sarcoma rarely metastasizes to other bones, so that its occurrence five years after the removal of the original growth is worthy of record, expecially from the viewpoint of differential diagnosis.

CASE REPORT

A married woman, aged 39, was admitted to the Neurosurgical Department, under the care of Mr. J. Hardman, complaining of a large spherical lump, the size of a half grapefruit, on the left side of the front of her head. It had been present for about six months. She had no other complaints but on direct questioning she said she had experienced some dizziness and occasional diplopia in the previous few months. She had three children, aged 16, 12, and 9 respectively. Her left leg had been amputated five years earlier for 'disease in the knee with some thickened bone'.

On examination there was a firm round bony swelling 6 in. in diameter over the left fronto-parietal region of the skull. It was smooth in contour and free from skin attachment. No bruit could be heard either over the lump or over the vault of the skull. There was gross bilateral papilloedema. No other abnormal physical signs were found, either in the central nervous system, or on general examination. The blood pressure was 140/90 mm. Hg. The left leg had been amputated at the upper third of the thigh and the stump appeared healthy and useful.

A radiograph of the skull showed an intensely sclerotic thickening of the bone in the left fronto-parietal region, interpreted as a large calcified meningioma. In the antero-posterior view the inner surface of the tumour was lobulated and it extended across the midline (Fig. 1). A left-sided percutaneous carotid angiogram (Figs. 1 and 2) showed marked displacement of the anterior cerebral artery across the midline with some downward displacement of the pericallosal and middle cerebral arteries. The tumour displaced the branches of the main vessels, compressing them together, but it did not appear vascular. The branches of the external carotid artery were elevated above the surface of the skull by this tumour. There was evidence of tentorial pressure coning. Although a radiograph of the chest was requested it had not been carried out by the time it was decided to remove the mass in the skull. This was undertaken as an urgent procedure owing to the patient's rapidly failing vision and the onset of vomiting.

OPERATION Under general anaesthesia with hypothermia, burr holes were made in the skull all the way round the tumour and across the tumour mass in the midline sagittal plane. The tumour was then removed by elevating the two bone flaps preserving the superior longitudinal sinus. The underlying dura was firmly adherent to the thickened part of the skull in several places and was diffusely infiltrated by nodules of hard calcified tumour. The infiltrated dura was removed with the bone and several nodules of growth had to be cut through, leaving a small residual amount of tumour which appeared to be invading the underlying brain. A few small nodules of growth were removed from the side of the longitudinal sinus. The defect in the skull was covered with bone from the deep freeze. The patient received 5 pints of blood which more than replaced her loss.

POST-OPERATIVE COURSE Her condition was satisfactory and she was recovering uneventfully till the eighth day

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FIG. 1

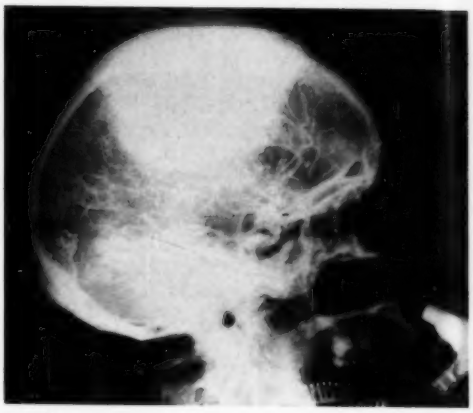

FIG. 2

FIG. 3

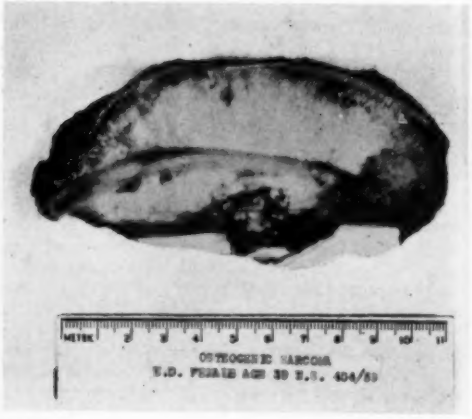

FIG. 4

FIG. 1. *Antero-posterior view of the skull showing lobulated bony tumour on the left side with displacement of vessels.*

FIG. 2. *Left percutaneous angiogram (lateral view) showing bony tumour displacing vessels.*

FIG. 3. *Lateral view of lower end of left femur showing osteogenic sarcoma.*

FIG. 4. *Sagittal section through tumour in the skull. Nodules of growth have penetrated the dura on the inner aspect.*

when she suddenly collapsed and died from what was thought to be a pulmonary embolus.

MACROSCOPIC APPEARANCE OF LEFT LEG (1954 SPECIMEN) The radiograph of the left femur showed a typical osteogenic sarcoma in its lower part (Fig. 3). The leg had been amputated through the upper third of the thigh and about 19 cm. of femur removed. The knee joint appeared normal but for about 10 cm. above the joint the femur was broadened and fusiform. A longitudinal section showed that the marrow was replaced for about 14 cm. to within 3 cm. of the cut end. The subperiosteal thickening of the bone was ivory hard.

SKULL (1959 SPECIMEN) The grossly thickened piece of bone removed from the left fronto-parietal region measured 13 × 12 cm. and was 5 cm. thick in its most affected part. The outer surface was smooth and protruberant while on the inner surface a large area of dura was firmly adherent. Many bony hard nodules of growth projected inwards through the dura. Some of these had a smooth contour, while others appeared roughened and had been cut through at operation. On sawing through the specimen the tumour was ivory hard and was present on both the inner and outer aspects of the vault as well as infiltrating it (Fig. 4). The outline of the vault could still be identified, being very distinct peripherally although rather faint in the centre. The bone of the infiltrated part of the vault was even harder than the tumour.

NECROPSY (21 HOURS AFTER DEATH) The body was that of an obese female with a healed large left frontal skin flap overlying a defect in the skull. This had been partially filled with bone grafts but the underlying swollen brain had herniated through the defect. There was no evidence of residual tumour in the vault of the skull, but several bony nodules of tumour were infiltrating the brain in the left frontal lobe towards the medial border of the hemisphere. The dura had been removed over an area, roughly 4·5 cm. in diameter, in this region. The left hemisphere was more swollen than the right with a tentorial pressure cone. There was ante-mortem thrombus in the superior longitudinal sinus.

Both lungs were studded with small, bony, hard nodules of metastatic tumour both on the surface and in their substance. There was an embolus in the right pulmonary artery at its bifurcation. As the pelvic and femoral veins did not contain any thrombus, the superior longitudinal sinus was thought to be the site of origin of the pulmonary embolus. Apart from a small cyst in the right ovary the other viscera were healthy. The bones of the spine and pelvis were sectioned but appeared free from metastases.

HISTOLOGY

LEFT FEMUR (1954 SPECIMEN) The tumour is made up of round or spindle-shaped cells which are laying down osteoid and bone throughout their extent (Fig. 5). The cells are arranged in small groups widely separated by the bony trabeculae. Several veins contain tumour cells both in their lumina and in clots adherent to their walls. No mitotic figures are seen.

SKULL (1959 SPECIMEN) The tumour has a similar appearance to that seen in the femur. The original bone of the skull is infiltrated throughout most of its extent and the marrow spaces are reduced to minute size by new deposition of bone with scanty scattered tumour cells. Externally the periosteum is elevated by a large mass of solid, well-formed bony trabeculae, often in parallel columns or in rounded masses separating small groups of tumour cells (Fig. 6). These cells are most numerous just beneath the pericranium, which they penetrate in several places. Internally, the tumour forms a large bony mass infiltrating and penetrating the dura. The tumour cells are uniform in appearance and no giant cells or mitotic figures are seen.

BRAIN Coronal sections through the left frontal lobe demonstrate that the residual tumour is confined mainly to the pia arachnoid, which is grossly thickened and compressing the underlying brain. In several places, however, the surface cortex is infiltrated (Fig. 7). The tumour in the pia arachnoid shows similar features to that seen in the skull, with numerous bony trabeculae between groups of tumour cells. Many of the arteries in the invaded pia arachnoid exhibit marked endothelial proliferation with narrowing of the lumen while several pial veins are occluded with fibrin thrombus. In the cortex and superficial white matter of this area is a wedge-shaped infarct, and several petechial haemorrhages have occurred into the softened tissue. The tumour penetrates the pial-glial membrane on the surface in several places and invades the necrotic cortex. It also extends into the perivascular spaces of the cortical vessels. There is a slight tendency for tumour cells to whorl in a few places. Nodules of tumour are present in the dura around the operation site. Tumour cells are seen infiltrating this membrane as well as being present on both its internal and external surfaces and invading its venous channels.

LUNG The nodules in the lung show a similar appearance with groups of tumour cells separated by much bone formation (Fig. 8).

OTHER ORGANS No metastases are seen in the viscera or bones sectioned.

DISCUSSION

REVIEW OF THE LITERATURE Willis (1952) reviewed the literature and collected only 11 reports of cases of sarcomas of bone metastasizing to other bones

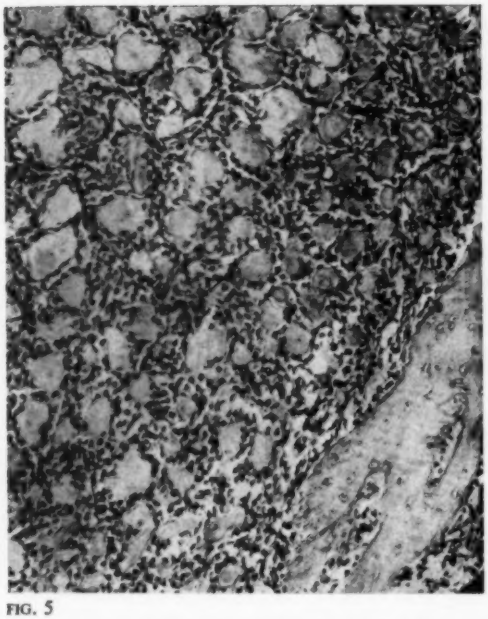

FIG. 5

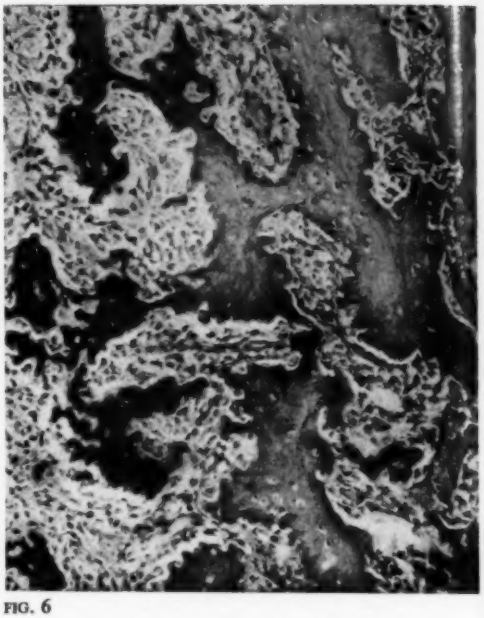

FIG. 6

FIG. 5. *Section of tumour in left femur showing diffuse osteoid formation. Haematoxylin and eosin × 100.*

FIG. 6. *Section of outer part of tumour in skull showing dense bony trabeculae and scanty tumour cells. Haematoxylin and eosin × 100.*

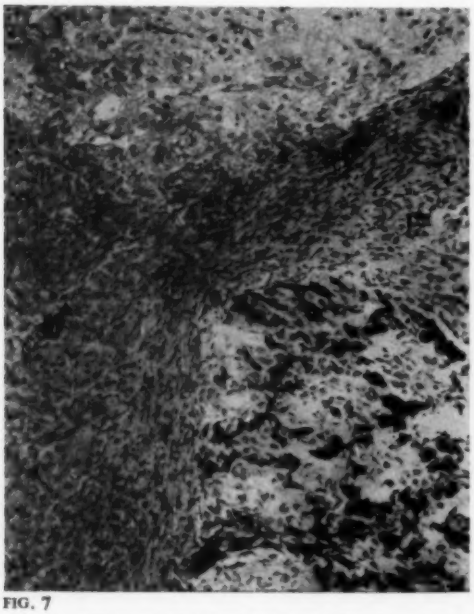

FIG. 7

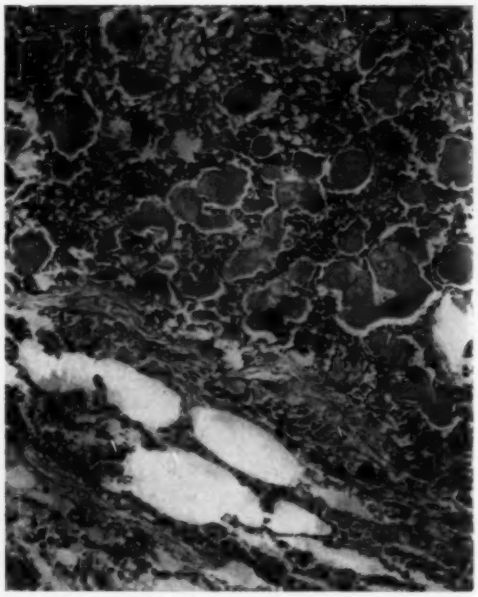

FIG. 8

FIG. 7. *Section of left frontal lobe showing tumour containing osteoid diffusely infiltrating pia arachnoid and invading the cortex. At the top of the section the pial-glial membrane is still intact. Haematoxylin and eosin × 100.*

FIG. 8. *Section of lung showing nodule of osteogenic sarcoma. Haematoxylin and eosin × 100.*

(Durham, 1883; von Recklinghausen, 1885; Nunokawa, 1908; von Roznowski, 1915; Roedelius, 1915; Joll, 1923; Martin, 1925 (2 cases); Bird, 1927; Dresser and Dumas, 1930; Dyke, 1931; Geschickter, 1932). To these 12 cases he added one of his own (Case 59).

Although White (1922) described a case of multiple pulsating bone tumours which he thought were probably alveolar sarcoma, the variability of the appearance suggesting either an epithelial growth or an angiosarcoma led to the omission of this case by Willis. A further 12 cases have been found in the subsequent literature.

In a series of over 500 cases of primary bone sarcomas, Geschickter (1936) found the cranial bones involved in only 12. In this series osteolytic sarcomas were twice as common as the sclerosing type. All except one of the patients in this series died of metastases but in only one case did these occur in bone, *viz.*, the spine and the ilium.

Silverman (1936) reported the case of a man aged 27 dying with multiple osteogenic sarcomata 11 weeks after the first symptom. Owing to the growths occurring almost simultaneously and showing similar appearances and stages of osteogenic development, a multicentric origin was postulated, especially as osteogenic sarcomas do not metastasize extensively to bone.

Ray and Galstaun (1938) reported a case with multiple bone tumours but their diagnosis was made on radiological grounds without histological confirmation.

Busso and Schajowicz (1945-6) recorded the case of an 8-year-old girl with sclerotic tumour foci in many bones without pulmonary involvement.

Two cases were reported by Ackerman (1948). They were both male aged 27 and 10. After the first bone tumour was recognized, other bone lesions developed at sites characteristic of primary osteogenic sarcoma. Thus only the metaphyseal ends of the shafts were involved, the rest being intact. As metastases from other types of tumours are encountered in other parts of the shafts of long bones, their absence in these two cases together with the simultaneous appearance of bone lesions and their analogous stages of osteogenic development were regarded as supporting Silverman's idea of multicentricity, although insufficient to prove it.

Halpert, Russo, and Hackney (1949) regarded the numerous bone sarcomata seen in a child aged 8 as being of multicentric origin. The history was very short as death ensued within four and a half months of the initial symptoms. The multicentricity of origin was supported by Lichtenstein (1952), who described two further cases.

Finlayson (1953) recorded a case with multiple bone and pulmonary tumours in a girl aged 12. Death occurred 20 months after the onset of symptoms. He pointed out that even in the cases with a very short history in which multicentricity of origin was suggested, the extremely rapid development of haematogenous skeletal metastases from one initial growth could not be excluded.

Moseley and Bass (1956) stressed the characteristic radiological appearances of the sclerosing type of osteogenic sarcoma, reporting a case of multiple bone tumours in a girl of 5, dying four months after the initial symptoms. No decision as to the explanation of the multiple growths was reached but Price and Truscott (1957) favoured the multicentric origin in their case.

METASTATIC ORIGIN OF SKULL TUMOUR Apart from the sarcomata arising in bones affected by osteitis deformans, where the frequency of association has been reported as varying between 0·9 and 30% (Porretta, Dahlin, and Janes, 1957), with the majority of authors reporting an incidence of 7 to 11% (Esposito and Berne, 1960), tumours considered to be of multicentric origin are very rare, occurring in young patients with a relatively short history (four weeks to 19 months). Even when the multiple bone tumours develop almost simultaneously, however, early haematogenous spread cannot be excluded. In the case reported in this paper the haematogenous spread cannot be doubted in view of the widespread invasion of blood vessels in the initial growth and the presence of pulmonary metastases. There was no evidence of Paget's disease, which is the commonest basis for bone sarcoma in the skull.

Although the majority of metastases of osteogenic sarcoma appear within two years of the onset (Willis, 1953), much longer periods may elapse, *e.g.*, 13 years in one of the cases of Badgley and Batts (1941). The osteoblastic or sclerosing types of osteogenic sarcoma (classification of Geschickter and Copeland, 1936) usually has the best prognosis and the interval of five years between the amputation and the appearance of the skull mass was sufficiently long to suggest other pathological lesions in the differential diagnosis of the present case.

DIFFERENTIAL DIAGNOSIS A meningioma was considered a likely possibility when the patient was first seen, especially in view of the rarity of multiple osteogenic sarcoma and the absence of Paget's disease. The radiological appearances were striking, and not typical of an osteoblastic meningioma. In the hyperostosis associated with meningioma, bone is laid down in parallel trabeculae close to the tables of the vault to form both an enostosis and exostosis,

and from these columns of bone radiate inwards and outwards with an external cap of a thin layer of fluffy bone (Rowbotham, 1939). The irregular, fusiform and rather fluffy thickening contrasts markedly with the densely sclerotic mass seen in the present case. The intense sclerosis, giving the impression of a snowball thrown into the calvarium, is characteristic of this type of osteogenic sarcoma. The lobulated inner border extending across the midline, as seen in the antero-posterior radiograph, also contraindicated a meningioma.

Although the morphological character of meningioma cells and osteoblasts is very similar (Russell and Rubinstein, 1959), there were histological features in the present case differing from those of a meningioma. Thus the tumour cells were small, round, or spindle-shaped, with fairly well-defined borders arranged in small groups in contrast with the larger, more spheroidal, poorly defined cells arranged in sheets in the endotheliomatous type of meningioma. In only a few places was there any tendency for the cells to form compact whorls and these were separated by osteoid formations. Although the latter superficially resembled psammoma bodies in several areas, the majority were devoid of concentric lamination. In bone invaded by meningioma, groups of endothelial cells, frequently in compact whorls, can often be seen diffusely ramifying the wide medullary spaces between the trabeculae (Cushing, 1922; Phemister, 1923). In contrast, in the case described, the affected part of the vault, which was harder than normal, showed intense new bone formation causing reduction of the spaces between trabeculae to minute channels usually devoid of cells.

My thanks are due to Mr. J. Hardman, F.R.C.S., for allowing me full access to his clinical notes and for the details of the operation. His helpful suggestions and advice are gratefully acknowledged. I should also like to thank Professor D. H. Collins for his interest, useful criticism, and help with the text; Dr. J. L. Edwards for his description of the original specimen and loan of the histological section; Dr. J. L. A. Grout for the radiographs of femur, and Dr. J. Wilkie for the cerebral angiograms.

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Pathological changes in temporal arteries removed from unselected cadavers

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SYNOPSIS Progressive degenerative changes in ageing temporal arteries are described. These changes are usually severe in elderly patients, hence their presence in diagnostic biopsies provides no evidence of temporal arteritis past or present.

In two patients in the series of random necropsies examined there were changes in the temporal arteries identical with those seen in patients known to have suffered from temporal arteritis in the past; in both these cases there was, in addition a giant cell aortitis.

The distinction between healed arteritis and degenerative changes due to age in a single biopsy specimen may be difficult. Furthermore, it is possible that steroid treatment may arrest the progress of the disease so that gross intimal thickening and vascularization of the artery wall do not occur.

Giant cell arteritis is not a rare disease. Two examples were discovered in 39 random necropsies on patients over 60 years of age.

During the examination of temporal artery biopsies it was noticed that those from patients with clinically typical temporal arteritis almost invariably showed active giant cell arteritis, but that there was rarely any histological evidence of active arteritis if the clinical diagnosis was in doubt. However, arteries from patients in this latter group usually showed 'degenerative' changes, frequently so severe as to raise the possibility of damage due to a previous attack of arteritis.

Temporal arteritis is a disease of the elderly, hence artery biopsies from possible cases might be expected to exhibit changes due to age. In order to assess the significance of such changes in biopsies and to distinguish them, if possible, from true healed temporal arteritis the temporal arteries from 60 cadavers were examined; these included both hospital and coroners' cases. The main arteries and a few centimetres of their anterior and posterior branches were removed. Three blocks were prepared from each artery so as to include the main stem and its major branches together with smaller branches if possible. Sections were stained with Ehrlich's haematoxylin and eosin and with Weigert's elastin stain. The series was divided into three age groups. The findings in each group are described below.

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RESULTS

YOUNG ADULTS (SEVEN PATIENTS AGED BETWEEN 24 AND 36 YEARS) Some of the medium-sized arteries (main stem and large branches) in every case showed a minor degree of intimal thickening. There was either duplication of the internal elastic lamina and an increase in collagen together with some smooth muscle in the intima (Fig. 1), or thickening by numerous thin elastic laminae, collagen fibres, and some smooth muscle cells. Thickening of the latter type was often distributed in plaques which were frequently conspicuous adjacent to the orifices of small branches, *i.e.*, points where atherosclerosis is common (Fig. 2). The internal elastic lamina was usually intact; if there were breaks they tended to occur next to a plaque of intimal thickening. A new elastic lamina was often developed adjacent to the endothelium overlying such plaques.

40-60 AGE GROUP (14 PATIENTS) Intimal thickening of the same types was more conspicuous and affected both large and small arteries. In seven patients there was evidence at necropsy of hypertensive cardiovascular disease, but the intimal thickening was not significantly greater in these. In a few arteries small atherosclerotic plaques were present. These were formed by rather granular elastic fibres interlaced with collagen fibres and contained some foamy

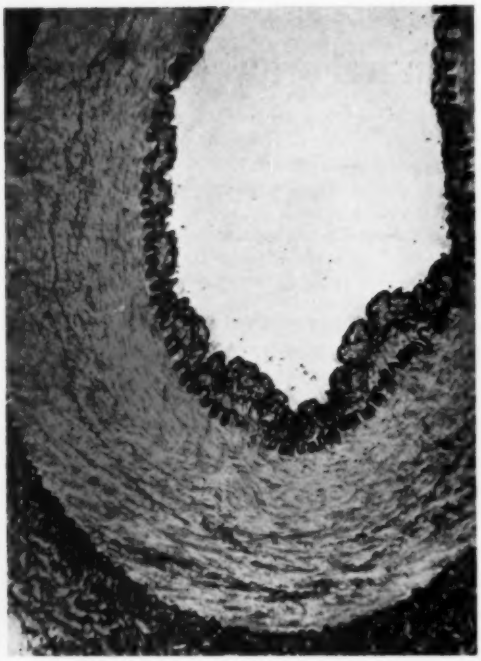
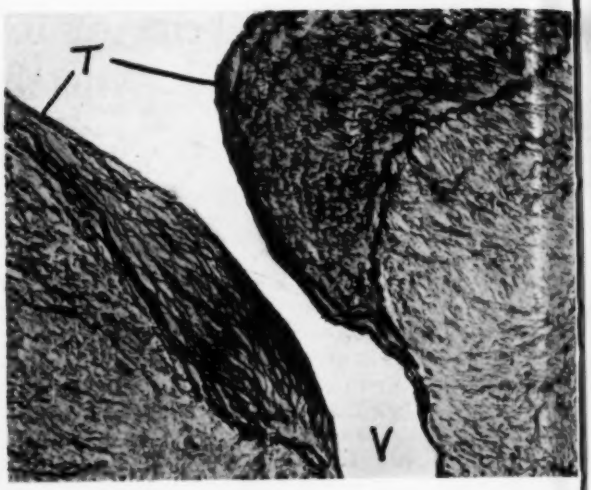


FIG. 1



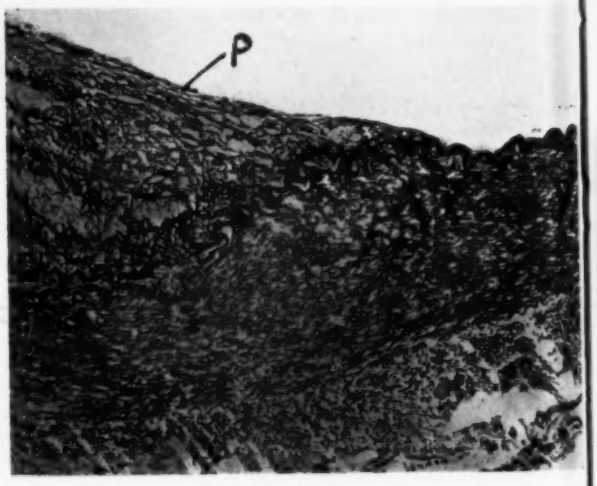


FIG. 3

FIG. 1. *Temporal artery from a male aged 24 years (Weigert's elastin stain × 85).*

FIG. 2. *Plaques of intimal thickening (T) adjacent to the orifice of a small branch (V) (Weigert's elastin stain × 140).*

FIG. 3. *An atheromatous plaque (P) in a temporal artery (Weigert's elastin stain × 110).*

macrophages (Fig. 3). 'Stretching' and fragmentation of the internal elastic lamina was often present beneath these plaques. The media was usually well defined and composed of circular muscle, except beneath some of the intimal plaques where the muscle fibres sometimes appeared to be longitudinal and continuous with smooth muscle in the intima through breaks in the internal elastic lamina.

Lesser degrees of the degenerative changes in the internal elastic lamina and of the associated fibrosis seen in the over-60 group were found in some of the arteries in this group.

OVER-60 GROUP (39 PATIENTS) Thirteen patients in this group were aged 80 or more years. The intimal thickening tended to be more marked, though not in every case, and its degree was not related to hypertension. It usually consisted of numerous concentric thin elastic membranes, often showing considerable fragmentation together with

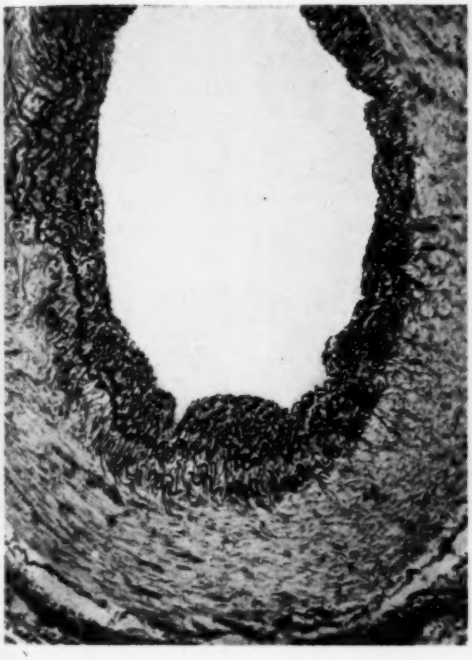


FIG. 4a. *Temporal artery from a female aged 79 years (Weigert's elastin stain × 85).*

FIG. 4b. *Temporal artery from a male aged 67 years. Note fibrosis in relation to the degenerating internal elastic lamina (Weigert's elastin stain × 85).*

some collagen and smooth muscle. Degenerative changes in the internal elastic lamina were the most important feature of this group, and were present to some extent in every patient. These changes included fragmentation, partial loss, and a change in staining properties (Figs. 4a, 4b). Often the internal elastic lamina was more eosinophilic than normal and had lost much of its black-staining properties with Weigert's elastin stain, appearing as a rather swollen, often fragmented, greenish-yellow refractile membrane. This last change was very common in patients over 80 years of age. In two patients patchy calcification of the degenerating membrane was observed. A variable degree of fibrosis occurred in relation to the degenerating membrane and was seen as dense, often hyaline, collagen which extended into the media and adjacent intima. Sometimes these collagen fibres appeared to have a radial arrangement and were associated with longitudinal smooth muscle. The media might show generalized loss of muscle with replacement fibrosis, and in a few arteries medial calcification was present. Inflammatory cellular infiltration (mainly lymphocytes in the adventitia) and vascularization of the walls of senile arteries (except in the two patients cited below) only occurred in relation to large atherosclerotic plaques; these were uncommon, being present in only one pair of arteries.

In two patients, aged 85 and 86 years, the histological appearances of the temporal arteries (both large and small branches) differed from those in the arteries of the corresponding age group. The changes were bilateral and comprised extensive loss of the internal elastic lamina, elastin preparations showing a few black granular fragments or a layer of diffuse granular material in its place; blood vessels, mainly capillaries, with conspicuous endothelial cells and some arterioles in the outer intima and inner media; considerable intimal thickening by concentric collagen fibres, rather granular elastic fibres, and numerous smooth muscle cells, the smooth muscle frequently forming a well-developed circular layer adjacent to the narrowed lumen; a new elastic lamina just beneath the endothelium; moderate fibrosis in the media usually in relation to the damaged internal elastic lamina; fibrous thickening and increased

FIG. 5a. *Healed arteritis. Temporal artery from patient with aortic aneurysm (Weigert's elastin stain × 110).*

FIG. 5b. *Main stem of temporal artery from same patient as in Fig. 5a (Weigert's elastin stain × 100).*

vascularity of the adventitia, with fragmentation and loss of the external elastic lamina (Fig. 5a, 5b). These changes were thought to provide evidence of healed arteritis. Reference to the necropsy findings revealed that the first patient died from myocardial infarction and histological examination showed an active giant cell aortitis and healing arteritis in small branches of the coronary arteries. The second patient had ruptured a fusiform aneurysm of the descending thoracic aorta. In sections of the aorta there was evidence of a late stage of giant cell aortitis and the anterior descending branch of the left coronary artery showed healing giant cell arteritis. The aorta was examined histologically in 29 other patients in this series; in none was there any evidence of giant cell aortitis.

DISCUSSION

The clinical and pathological features of temporal (giant cell) arteritis were first described by Horton, Magath, and Brown (1932, 1934) and numerous examples have since been reported (Roux, 1954). It is now recognized that the disease is not as rare as was formerly believed (Robertson, 1952; Cameron, 1959).

The histopathological features of the active phase have been frequently described (Cooke, Cloake, Govan, and Colbeck, 1946; Harrison, 1948; Cardell and Hanley, 1951; Kimmelstiel, Gilmour, and Hodges, 1952; Lander and Bonnin, 1956), but much less attention has been paid to the appearances of healed arteritis and how these may be distinguished from degenerative changes commonly present in the temporal arteries of elderly patients. Kimmelstiel *et al.* (1952) compared the 'senile' changes in the temporal arteries of 50 elderly persons with the changes seen in temporal arteritis. At no time were senile degenerative changes associated with a giant cell reaction, a finding confirmed by the present results. They noted hyaline connective tissue in relation to the internal elastic lamina of senile arteries, a change they called 'perielastic hyalinosis'.

Inflammatory cellular infiltration of the artery wall was not seen in senile arteries, except occasionally in relation to large atherosclerotic plaques. A

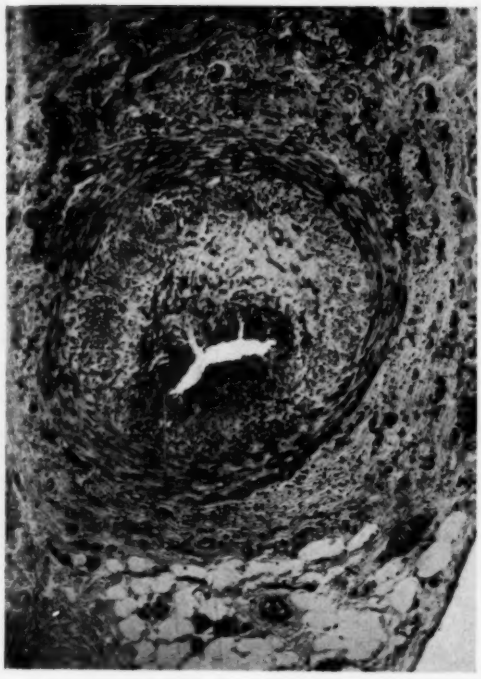


FIG. 6a. *Temporal artery biopsy from patient with history of temporal arteritis two years previously (haematoxylin and eosin × 85).*

FIG. 6b. *Same as Fig. 6a (Weigert's elastin stain × 85).*

study of biopsies from 22 typical clinical examples of temporal arteritis has shown that inflammatory cellular infiltration often persists until a late stage in the healing process. Even when inflammatory cells have completely disappeared an artery damaged by giant cell arteritis is likely to show certain histological features which differ from those present in 'senile' arteries, *viz.*, greater intimal thickening, often with a well-developed layer of smooth muscle adjacent to the lumen, vascularization of the artery wall, and considerable loss of the internal elastic lamina, surviving segments often appearing as a granular black layer in elastin preparations.

However, the distinction between healed arteritis and degenerative changes due to age in a single biopsy specimen may be difficult. Difficulty may also be experienced in studying patients suffering from giant cell arteritis who have received steroid treatment before biopsy is performed. Steroids are thought to reduce the oedema and inflammatory cellular reaction in giant cell arteritis (Harrison, Harrison, and Kopelman, 1955), and should this occur before gross intimal thickening and vascularization of the wall takes place, it is possible that the residual changes might be indistinguishable from those due to senile degeneration.

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The pathology of 111 cases of primary hepatic malignancy collected in the Liverpool region

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SYNOPSIS The clinical records and preserved specimens of 111 cases of primary malignant disease of the liver have been analysed. All were diagnosed in the Merseyside area, chiefly in Liverpool and Birkenhead, and all but nine were Europeans. Necropsy was done in 89.

The tumours consisted of 108 carcinomas, two sarcomas, and one mixed tumour. Liver cell carcinoma was nearly four times more frequent than carcinoma of the intrahepatic bile ducts. Both types were commoner in males, the difference between the sexes being greater in the case of liver cell carcinoma. Predisposing conditions included portal cirrhosis, haemochromatosis, and cystic malformations of bile ducts. The gross and microscopic appearances are described and the pathological effects discussed. Liver cell carcinoma tends to invade and permeate veins within the liver but seems, nevertheless, to remain limited to the liver for longer than carcinoma of the intrahepatic bile ducts. Liver cell carcinoma is more characteristic microscopically. The commonest occupation among the male cases was seafarer.

The files of the Liverpool Cancer Control Organization for the years 1947 to mid-1959 were found to contain entries of over 100 cases of primary liver cancer. Because geographical factors are important in this disease, and since most of our information about primary cancer of the liver is derived from material collected in Africa (Symposium, 1957), or America (Edmondson and Steiner, 1954; Edmondson, 1958), it seemed worth undertaking a study of the Liverpool cases.

About a dozen cases were discarded because the material available was insufficient to substantiate the diagnosis, but sections or preserved tissue with short clinical or necropsy notes were available in 111 cases. The results of a preliminary classification of these cases are given in Table I.

TABLE I

111 CASES OF PRIMARY LIVER MALIGNANCY

Type of Tumour	*No. of Cases*	*Sex*	
		Male	*Female*
Carcinoma	108	82	26
Sarcoma	2	0	2
Mixed	1	0	1

The rare mixed and mesodermal tumours may be dismissed briefly.

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MIXED AND MESODERMAL TUMOURS

The mixed tumour was in a 3-month-old baby girl and caused abdominal distension, raising of the diaphragm, and splaying of the ribs. The tumour, which was in the right lobe, had not metastasized but caused death, in the opinion of the pathologist who did the necropsy, by compressing the inferior vena cava to cause profound disturbance of the circulation. Microscopically there were large areas of well-formed bone, myxomatous areas containing haemopoietic cells, and adenocarcinomatous areas. Tumours of this type are well recognized and selected examples from amongst the published cases have been used by Edmondson (1958).

The two patients classed as having sarcoma were both women in early middle age. In one case the diagnosis was made by biopsy of the hepatic mass during a laparotomy for acute symptoms attributable to haemorrhage from the tumour. The microscopic diagnosis was angiosarcoma. There was no necropsy.

The other patient also had a sarcoma of blood vascular tissues and both the liver and the spleen were affected. The woman had seemed to be in good health but died suddenly and at necropsy was found to have haemoperitoneum from rupture of a hepatic tumour that was diagnosed microscopically as a haemangioblastoma. The appearances suggested that malignancy had been superimposed upon congenital

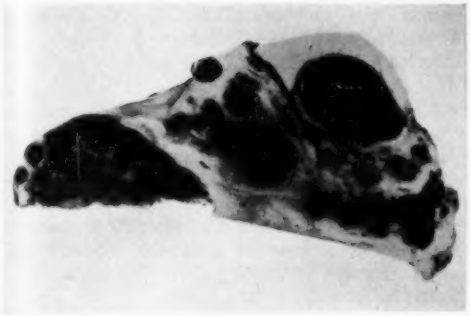


FIG. 1

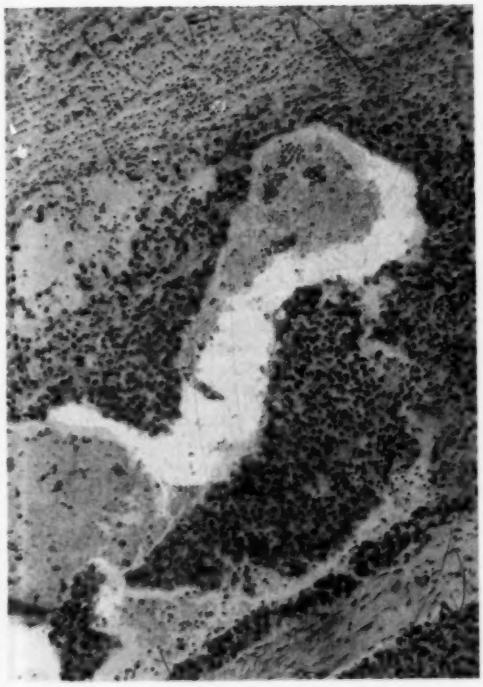


FIG. 2

FIG. 1 *Macroscopic appearance of part of a liver almost completely replaced by malignant haemangioblastoma.*

FIG. 2. *Low-power view of malignant haemangioblastoma. Haematoxylin and eosin* × *80.*

vascular malformations in the liver. The splenic tumour was similar and may have been metastatic, or a result of simultaneous malignant change in malformed splenic blood channels. Figs. 1 and 2 show the macroscopic and microscopic appearances of parts of the tumour. Cases of this type have been described by Hastings-James (1949), Baker, Paget, and Davson (1956), and Edmondson (1958).

CARCINOMA

Table II shows that in 20 of the 108 cases diagnosed

TABLE II

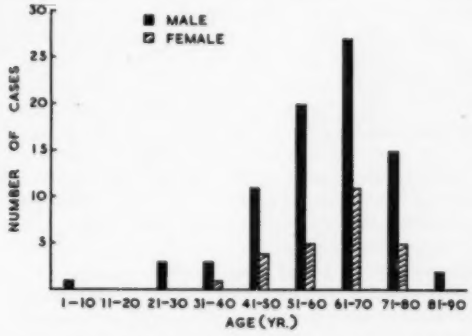
CASES OCCURRING IN 1947-59

Diagnosis by	*No. of Cases of Carcinoma*	*Sex*	
		Male	*Female*
Necropsy	88	69	19
Biopsy	20	13	7
Total	108	82	26

as carcinoma there was no necropsy. Most of these cases, however, had had a laparotomy, had no clinical evidence of a primary growth elsewhere, and had biopsy appearances compatible with primary carcinoma of the liver. The diagnosis 'carcinoma' includes liver cell carcinoma and carcinoma of intrahepatic bile duct epithelium (cholangiocarcinoma).

SEX Male cases predominated by slightly more than 3 to 1. The greater liability of men to the predisposing conditions of portal cirrhosis and haemochromatosis accounts largely, but perhaps not entirely, for the differing sex incidence. The sex difference was much less in the cases classified histologically as cholangiocarcinoma (Table V).

AGE In both sexes the incidence was highest in the sixth decade but the range was greater in males.



The male cases included one of liver cell carcinoma in a boy 1 year 11 months old, and two cases of men in the ninth decade, one aged 80 and the other 88, both with liver cell carcinoma. The diagram illustrates the age distribution of the two sexes.

RACE All the female cases were European. The male cases included nine non-European, of whom four were West Africans, two Indians, two Chinese, and one West Indian of African stock.

OCCUPATION No occupation had been recorded in the notes of 22 of the cases. The occupation of 18 of the 26 female cases was entered as 'housewife'. The commonest occupation amongst the male cases was 'seafarer'. There were 17 cases in this group, including one ship's master, one officer, and four stewards. The group included seven of the nine non-European cases. Other occupational groups were as follows: labourers 10, clerical 10, engineers 4, police 3, painters 3, army and R.A.F. 2. The remaining 22 cases had occupations, no two of which were similar.

INCIDENTAL CONDITIONS Gall stones were present or had been removed in 11 cases. Active peptic ulcers or the scars of healed ulcers were recorded in nine cases. A history of syphilis or positive serological tests for syphilis were recorded in six cases.

PREDISPOSING LESIONS The cases with, and without lesions suspected of predisposing to hepatic carcinoma are tabulated in Table III. No cases of infestation by *Clonorcis sinensis*, whose relationship to carcinoma of small bile ducts has been demonstrated by Hou (1956), were recognized. The lesions considered to be important are shown in Table III.

TABLE III

PRESENCE OR ABSENCE OF PREDISPOSING LESIONS IN 108 CASES OF CARCINOMA

Lesion	*No. of Cases*	*Sex*	
		Male	*Female*
Portal cirrhosis	58	51	7
Haemochromatosis	7	7	0
Banti's syndrome	1	0	1
Cystic malformation of bile ducts	2	0	2
No predisposing lesion	40	24	16

'*Portal cirrhosis*' This included all types of fibrous disorganization of structure with the exception of the fibrosis of haemochromatosis and biliary cirrhosis. No cases of Wilson's disease were recognized. In a few of the cases alcohol may have been important but in most of the cases the records contained either no entry of the patient's alcohol consumption or indicated temperate habits. No attacks of infective hepatitis were recorded in the case histories.

'*Haemochromatosis*' The criteria for inclusion in this group were fibrosis of the liver and loading of the liver cells with haemosiderin pigment whose presence could not be explained by previous blood transfusions or episodes of haemolysis. Only one of the seven cases had had the diagnosis established before necropsy. This was a man, 65 years old at the time of his death, who had attended a diabetic clinic where he was found to have a pigmented skin and an easily palpable spleen as well as diabetes. Puncture biopsy of the liver established the diagnosis of haemochromatosis. Death was due to a combination of pyaemic infection and diabetic ketosis. At necropsy a small primary carcinoma of liver cells was an incidental finding in the liver along with marked fibrous haemochromatosis.

In another case the haemochromatosis and carcinoma of the liver were incidental findings in a man who had died of coronary arterial occlusion. He too had splenomegaly. In a third case the haemochromatosis and liver tumour were incidental necropsy findings in a man of 78 who had died of cerebellar haemorrhage. The urine had not been examined before or after death.

Of the remaining four patients, there were two whose urine was recorded as negative for sugar, the others had no record of an examination of the urine. All four had been diagnosed clinically, or at laparotomy, as having cirrhosis of the liver and in one pigmentation of the skin was recorded. This man died of haemorrhage into the peritoneum from the tumour of the liver. One other died from haemorrhage from oesophageal varices, while the other two died of combinations of cachexia and hepatic insufficiency.

All the cases were men, the youngest 57 and the oldest 78. One liver cell carcinoma was in the left lobe; all the other tumours were in the right lobe. One of the tumours was an adenocarcinoma of intrahepatic bile duct epithelium, all the others were liver cell carcinomas. No iron was detected in any of the tumour cells by Perls's reaction despite the strong Prussian blue reaction of adjacent non-neoplastic liver cells.

The cholangiocarcinoma had invaded lymph nodes near the liver and one of the liver cell carcinomas had invaded the right side of the diaphragm and the right kidney by direct extension. No tumour was found beyond the liver in the other cases.

'*Banti's syndrome*' Carcinoma of the liver in association with the Banti syndrome is rare and the following case is an unusual one.

A married woman of 55 noticed a 'numbness in her stomach' about a year before her admission to hospital. About six months later the abdomen and ankles began to swell and this was followed by attacks of pain in the left hypochondrium, sometimes quite severe, though usually lasting for only a few minutes. On her admission to hospital she had slight jaundice with a palpable spleen

and signs of free fluid in the abdomen. Haemoglobin was 11·4 g. (77% of normal), red cells 3·0 million, platelets 20,000, and total white cells 800 per c.mm. (50% neutrophil polymorphonuclears, 46% lymphocytes, and 4% monocytes). Radiologically oesophageal varices were present. During her stay in hospital the white cells increased somewhat but the ascites became worse and had to be tapped. The surgeons were unwilling to undertake any operative treatment and death was the result of haemorrhage from the oesophageal varices. At necropsy the spleen weighed 1,568 g. The liver was scarred and shrunken, weighing only 672 g. Two rounded nodules of necrotic bile-stained tumour were present in the right lobe and microscopically these were areas of bile-secreting liver cell carcinoma.

As the blood picture was that of 'splenic anaemia', and the spleen much enlarged clinically and pathologically, with death from rupture of oesophageal varices, this case can be classified as an example of the Banti syndrome. The severe scarring and shrinkage of the liver suggests that the Banti syndrome may have been preceded by an episode of liver necrosis but no hint of any such episode could be found in the case history.

'*Cystic malformations of bile ducts*' In two women congenital cysts of the liver were associated with carcinoma of the intrahepatic bile ducts. Both were surgical cases without necropsy confirmation of the diagnosis.

The first case was a 30-year-old housewife who had a laparotomy on 2 October 1950 because of a lump which had been present in the upper abdomen for some months. A cyst was found in the liver. Excision was not attempted but biopsy was carried out. There was a wall of fibrous tissue lined by epithelium that appeared to be biliary. The contents of the cyst were purulent. The patient's general health remained good after the operation and when she was examined eight months later there had been no increase in the palpable mass. Partial hepatectomy was then attempted but had to be abandoned because the tumour was found to have involved the abdominal wall, the diaphragm, and the peritoneum. On biopsy the tumour was found to be a poorly differentiated carcinoma. About 12 days later the patient had the first of a series of haematemeses from which she died.

The second case was a 68-year-old housewife who had indigestion, jaundice, and an enlarged liver. At laparotomy the liver was enlarged by a tumour and contained many small cysts. Cysts were also present in the pancreas. The gall bladder and extrahepatic bile ducts were normal. Biopsy samples of the liver and a lymph node were diagnosed as carcinoma of the intrahepatic bile ducts associated with cystic malformations of the ducts. Secondary carcinoma was present in the lymph node. At the time of her death, two months later, there were radiological signs of secondary tumour in her spine.

Both cases are similar to examples reported by Willis (1943) and by Richmond (1956).

PATHOLOGY OF PRIMARY CARCINOMA OF THE LIVER

ASCITES This was present in 61 of the cases (56·5%).

JAUNDICE This was present in 44 of the cases (40·7%).

SPLENOMEGALY A minor degree of splenic enlargement, up to about 500 g., was relatively common but enlargement likely to be of clinical significance was present in only four cases. Of these two had haemochromatosis, and one had the Banti syndrome.

MACROSCOPIC APPEARANCES The presence or absence of cirrhosis was very important in determining the naked-eye appearances. In the absence of cirrhosis a nodule or mass of pale or slightly bile-stained tumour was easily distinguished from the adjacent liver and a provisional diagnosis of primary cancer of the liver could be made if no primary tumour could be found elsewhere. In the presence of cirrhosis the carcinoma was sometimes obvious as white, firm, infiltrating tumour but in some cases the differentation between nodules of liver cell carcinoma and nodules of regenerative hyperplasia of liver cells was sometimes very difficult. In one case the only hint of the presence of carcinoma was that the liver weighed 4·3 kg. After the true state of affairs had been recognized microscopically, inconspicuous invasion of veins could be seen when the cut surface was scrutinized carefully. Fig. 3 is a photograph of a slice of this liver.

In general the carcinomatous nodules tended to be paler than purely regenerative nodules (Fig. 4), and in haemochromatosis the pallor of the tumour against the rusty brown background of the liver was striking. Sometimes invasion and permeation of veins, with or without thrombosis, was obvious to the naked eye (Fig. 5). Invasion of veins was more common in liver cell carcinoma. When there was no invasion of veins and no obvious bile secretion by the tumour even a tentative differentiation between liver cell carcinoma and cholangiocarcinoma was almost impossible macroscopically. Fig. 6 illustrates the naked-eye appearances of a cholangiocarcinoma.

MICROSCOPIC APPEARANCES Liver cell carcinoma could usually be diagnosed more confidently than cholangiocarcinoma, chiefly because it was more difficult to be sure that a cholangiocarcinoma was not a secondary tumour. In the case of liver cell carcinoma the most characteristic pattern was the so-called solid trabecular type illustrated in Figs. 7 and 8. Bile secretion was an excellent indication of

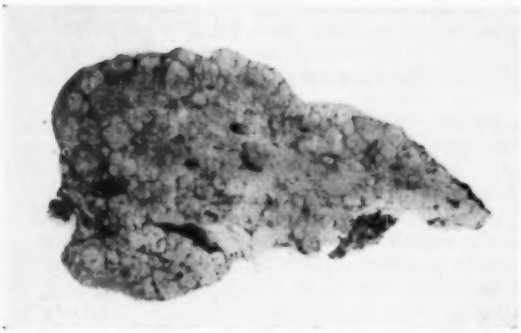


FIG. 3. *Nodules of liver cell carcinoma resembling nodular regenerative hyperplasia.*

FIG. 4. *Pallor of nodule of liver cell carcinoma.*

FIG. 5

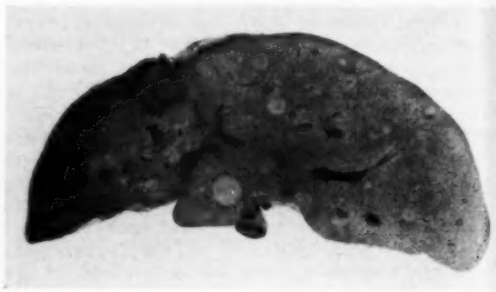


FIG. 6

FIG. 5. *Invasion of hepatic veins and inferior vena cava by liver cell carcinoma.*

FIG. 6. *Cholangiocarcinoma of the left lobe of a non-cirrhotic liver with secondary nodules throughout the right lobe.*

the nature of the tumour cells and was sometimes present in tumours in which the malignant cells had lost almost all resemblance to liver cells. Fig. 9 illustrates such a tumour which resembled thyroid gland except for bile staining of the secretion in the acini. Other features of liver cell carcinoma were multinucleated giant cells (Fig. 10) and a special tendency to penetrate and permeate veins (Fig. 11). Vacuoles of glycogen or fat were also taken to indicate an origin from liver cells. In many of the cases, however, no material for frozen sections was available. In some cases there was very marked variation in the appearances from place to place (Figs. 12 and 13). Such variation was taken to be evidence in favour of an origin from liver cells because of the tendency for such tumours to form gland-like structures (Steiner, 1959).

Cholangiocarcinoma usually had a glandular structure, often papillary (Fig. 14) and sometimes had mucus-secreting, signet ring cells (Fig. 15). The picture was similar to that of carcinoma of the extrahepatic bile ducts except that large accumulations of extracellular mucus were not seen. One case that was forming small cholangiole-like tubules (Fig. 16) was probably of the type that Steiner (1959) has named the cholangiolocellular type.

Table IV indicates how the cases of carcinoma were classified histologically. The seven tumours not assigned to either category were anaplastic and without characteristic features. Liver cell carcinoma was considerably more common, 74·1% as compared with only 19·4% of cholangiocarcinomas. Both types were more common in men but the discrepancy

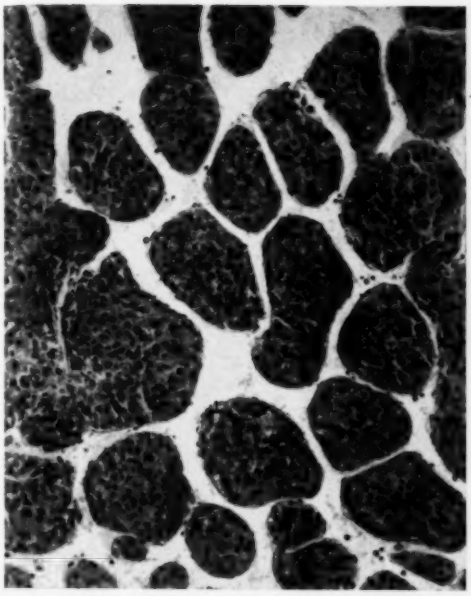


FIG. 7. *Solid trabecular pattern in liver cell carcinoma. Haematoxylin and eosin × 120.*

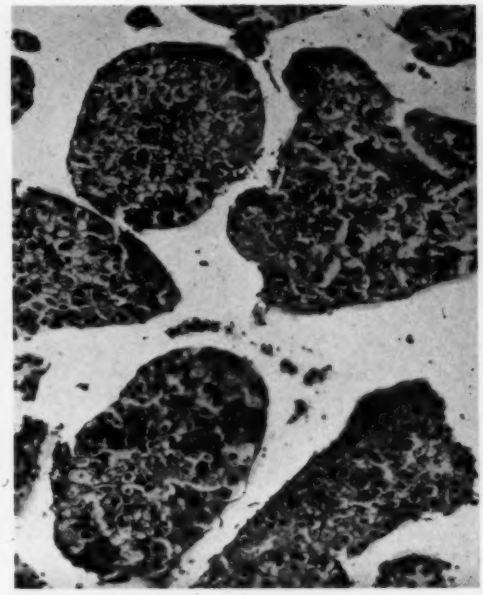


FIG. 8. *Solid trabecular pattern with vacuolation. Haematoxylin and eosin × 120.*

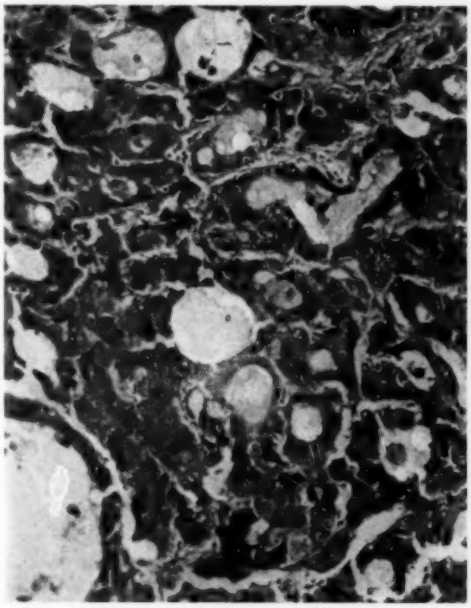


FIG. 9. *Thyroid-like structure of a bile-secreting liver cell carcinoma. Haematoxylin and eosin × 150.*

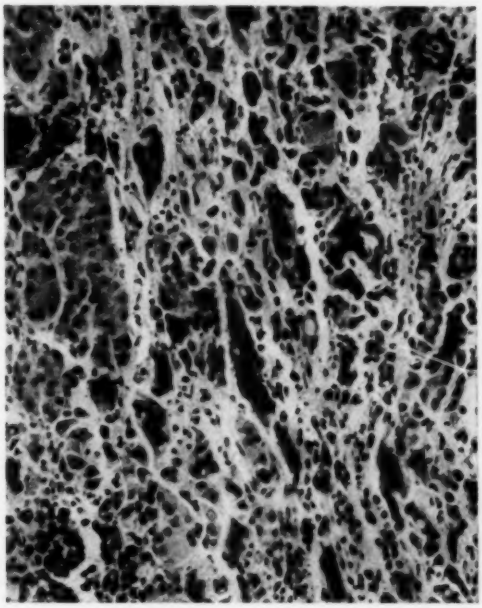


FIG. 10. *Multinucleated giant cells in liver cell carcinoma. Haematoxylin and eosin × 150.*

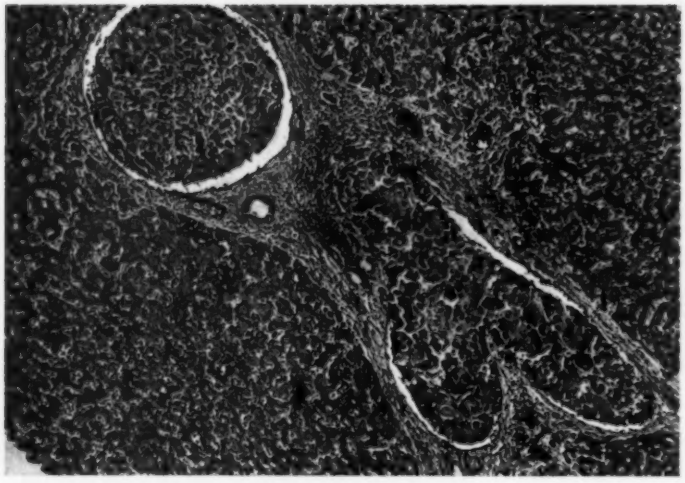


FIG. 11. *Permeation of veins within the liver by liver cell carcinoma. Haematoxylin and eosin × 68.*

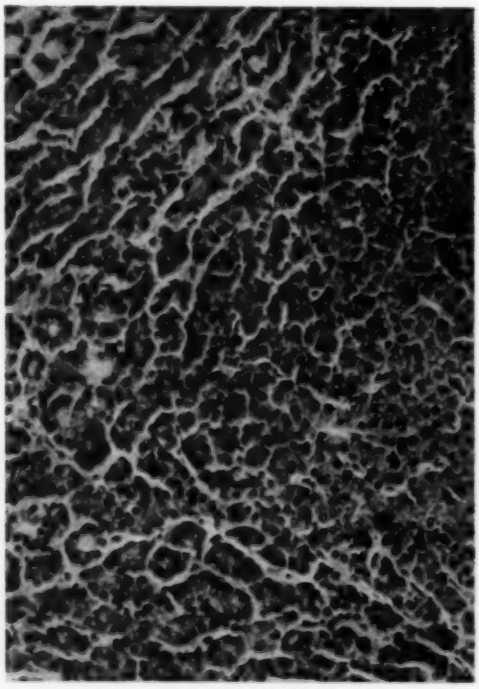


FIG. 12. *Well-differentiated area of liver cell carcinoma. Haematoxylin and eosin × 150.*

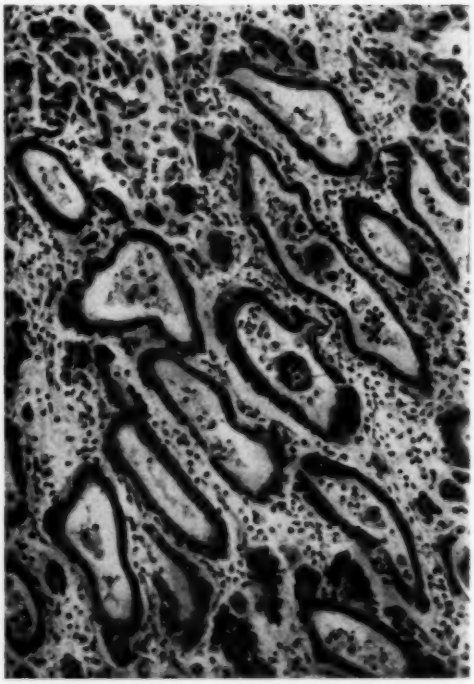


FIG. 13. *A different area of the same tumour shown in Fig. 12. Haematoxylin and eosin ×150.*

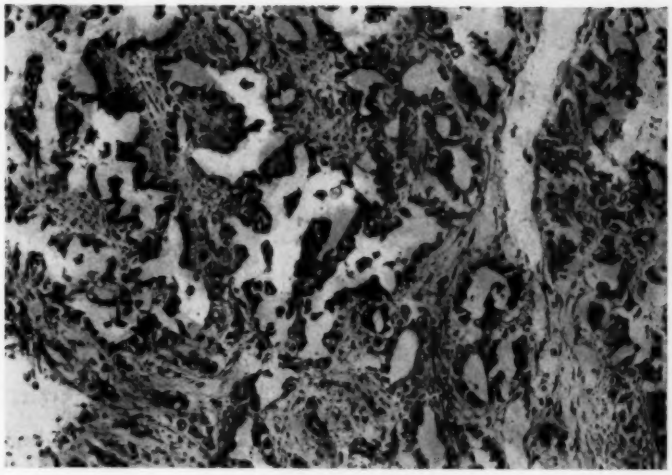


FIG. 14. *Slightly papillary adenocarcinomatous cholangiocarcinoma. Haematoxylin and eosin × 180.*

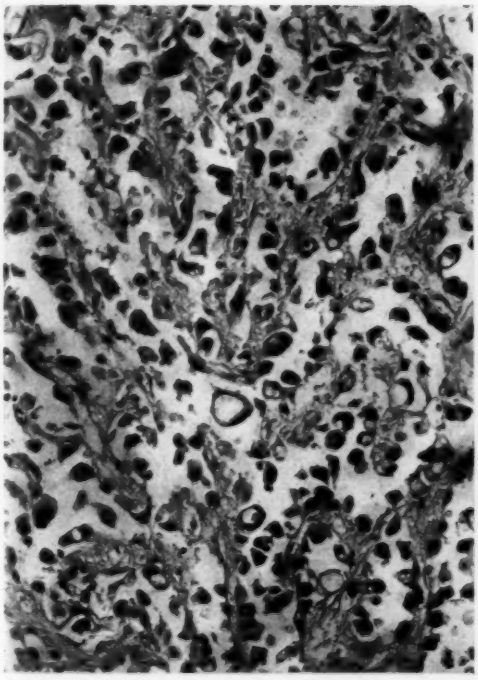


FIG. 15. *Signet ring cells in cholangiocarcinoma. Haematoxylin and eosin × 260.*

FIG. 16. *Cholangiole-like structure of carcinoma of intrahepatic bile duct epithelium. Haematoxylin and eosin × 41.*

TABLE IV

HISTOLOGICAL CLASSIFICATION OF 108 PRIMARY LIVER CARCINOMAS

Type	*Sex*	
	Male	*Female*
Liver cell	65	15
Cholangiocarcinoma	12	9
Indefinite	5	2

between the sexes was less in the case of the cholangiocarcinomas.

None of the tumours was classed as being a combination of the two types.

PRESUMED PRIMARY CARCINOID TUMOURS Amongst the tumours of bile duct epithelium there were two that may have originated from argentaffin cells. Tumours suspected to be primary liver tumours of this type are mentioned by Edmondson (1958). One of the Liverpool cases has been reported by Stanley (1958). The patient suffered from the 5HT syndrome and was found to have multiple hepatic tumours. No primary tumour elsewhere was found at necropsy.

The other patient died in 1949 before the 5HT syndrome had been recognized or associated with carcinoid tumours.

A woman of 62 attended hospital in January 1949, because of diarrhoea which she had had for five months. At first she had had two or three bowel motions per day, later the motions increased to five to 10 per day. On examination a swelling was seen in the right hypochondrium and right lumbar region. A barium meal, barium enema, excretion pyelography, and cystogram were negative apart from showing that the pylorus and hepatic flexure of the colon were depressed by a mass in the region of the gall bladder. At operation on 28 January 1949, a firm, rounded swelling, about 3½ in. in diameter, was found within the substance of the liver immediately deep to the attachment of the falciform ligament. The rest of the liver appeared healthy but the right lobe extended to well below the level of the right kidney. The tumour had a clear line of demarcation from the liver and was enucleated.

The patient made a good recovery from the operation but diarrhoea recurred within a month. For a time her general health improved and her weight increased but this was not maintained and when she was seen seven months after the operation a recurrent mass was felt in the liver beneath the operation scar. Diarrhoea was now very troublesome. She was not seen again but it was learned that she died about four months later. There was no necropsy.

The excised tumour was a lobulated yellowish mass with a smooth outline about 8 cm. in diameter with some cystic blood-filled spaces. Microscopically there were areas, especially at the periphery of the tumour (Fig. 17), where the appearances were those of a carcinoid tumour. Argentaffin granules were demonstrated by Hamperl's

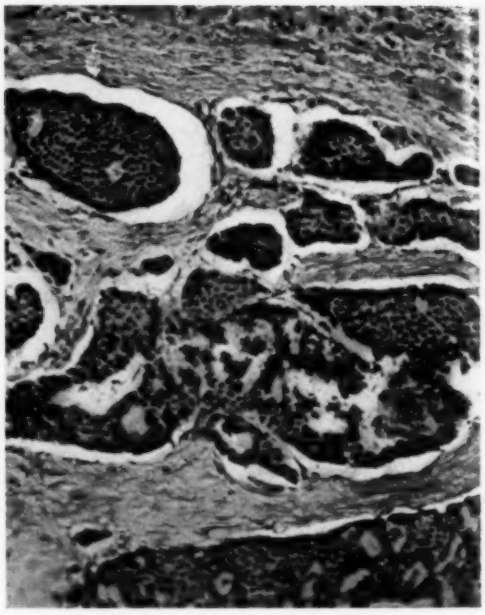


FIG. 17. *Carcinoid tumour of the liver, probably primary. Haematoxylin and eosin × 150.*

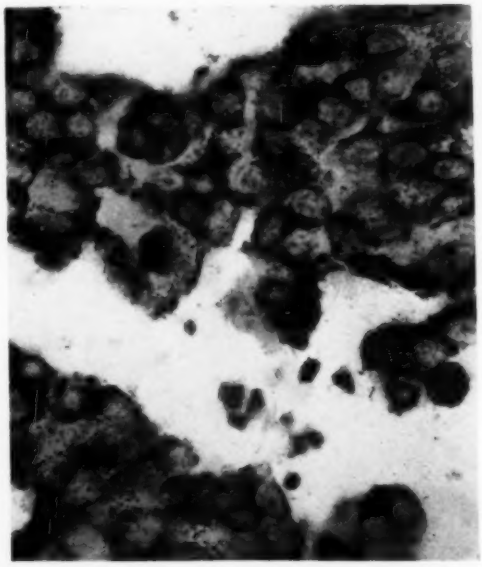


FIG. 18. *Argentaffin granules in carcinoid tumour of the liver. Hamperl's protargol × 1,600.*

(1952) protargol method (Fig. 18) and by the alkaline diazo reaction using fast red salt B.

This case can only be presumed to be one of primary carcinoid tumour of the liver. The surgical treatment was based on the belief that the tumour was primary in the liver. Throughout the illness diarrhoea was the main symptom but no cause within the bowel was found clinically or on laparotomy. The excision of the tumour produced temporary relief of the diarrhoea and its recurrence coincided with recurrence of the tumour. Apart from the diarrhoea no other features of the 5HT syndrome were noted but at that time the syndrome had not been recognized.

INVASION AND METASTASIS In some of the cases invasion of veins within the liver with superimposed thrombosis was very striking. So far as could be learned from the available information in the 88 necropsy cases the incidence of such thrombosis is given in Table V.

TABLE V

VENOUS THROMBOSIS WITH PRIMARY CARCINOMA OF LIVER

Hepatic	*Portal*	*Inferior Vena Caval*	*Elsewhere*
6	9	6	6

Fig. 19 illustrates thrombosis of a large branch of the portal vein. The six cases with thrombosis of veins far from the liver, usually in the legs, were probably examples of the venous thrombosis long known to be common in cases with carcinoma of abdominal organs (Trousseau, 1873).

As has been noted by others, invasion of veins is by no means invariably associated with blood-borne secondary deposits, and invasion of lymph nodes was the commoner type of spread in both liver cell carcinoma and cholangiocarcinoma. Moreover, although invasion of veins was more common in liver cell carcinoma, metastatic deposits were less common with this type of tumour than with cholangiocarcinoma.

DISTRIBUTION OF SECONDARY DEPOSITS Of the 88 necropsy cases, 84 could be classified into liver cell or cholangiocellular types. In the 67 liver cell carcinomas, metastasis had occurred in 22, approximately one third, while in the 17 cases of cholangiocarcinoma metastasis had taken place in 13, over three-quarters.

The organs affected were as follows:—

Liver cell carcinoma Lymph nodes in 12 cases, lungs in 10, diaphragm in 6, peritoneum in 4, adrenals in 3, bone in 2, pleura in 2, abdominal wall in 1, heart in 1, and thyroid in 1.

FIG. 19. *Thrombosis of the portal vein in a liver cell carcinoma in a cirrhotic liver.*

Cholangiocarcinoma Lymph nodes in 9 cases, adrenals in 7, lungs in 3, diaphragm in 2, pleura in 2, peritoneum in 2, bone in 2, and kidney in 1.

In both types the involvement of the diaphragm, abdominal wall, pleura, peritoneum, and, in some cases, the right adrenal, appeared to be by direct extension rather than by the lymphatic or blood systems.

INVASION OF THE BILIARY SYSTEM Edmondson (1958) describes invasion of the common bile duct by a liver cell carcinoma and could find only one report of a case of this type. Invasion of the common duct, with obstructive jaundice as result, was a feature of one of the cases in Liverpool.

An 80-year-old man had a laparotomy for jaundice and an enlarged liver. A cirrhotic liver was found and the gall bladder, which contained stones, was removed. 'Mud' was removed from the common hepatic duct and the duct was drained. The man died six weeks later, and at necropsy a primary liver cell carcinoma that had originated in the cirrhotic liver was found to have invaded and obstructed the common bile duct for a distance of 3 cm. The liver weighed 1,150 g. and there was thrombosis of the left hepatic vein with extension of the thrombosis to the inferior vena cava. The immediate

FIG. 20. *Invasion of a bile duct by liver cell carcinoma. Slightly enlarged.*

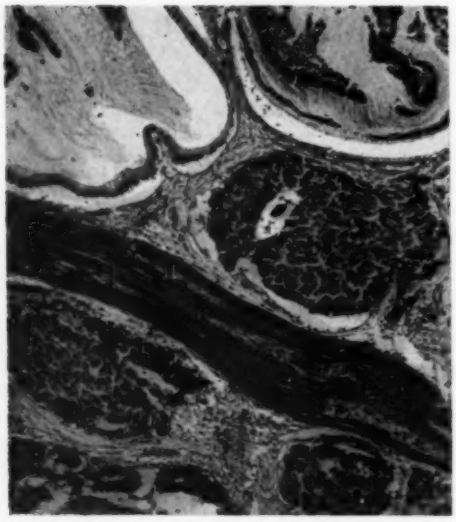


FIG. 21. *Wall of gall bladder being invaded by liver cell carcinoma. Normal mucosa with adherent bile and mucus is present on the left. Haematoxylin and eosin* × *80.*

cause of death was haemorrhagic pericarditis. No secondary deposits beyond the liver and biliary system were found.

Minor degrees of invasion of the biliary system were found in two other Liverpool cases. Fig. 20 shows a primary liver cell carcinoma invading a large bile duct, and Fig. 21 is of a section from another case in which the wall of the gall bladder is being penetrated by a liver cell carcinoma that arose in the non-cirrhotic liver of a 51-year-old woman and caused death by intraperitoneal haemorrhage.

HAEMORRHAGE ASSOCIATED WITH PRIMARY CARCINOMA OF LIVER Haemorrhage from oesophageal varices is a common manifestation of the disturbance of the portal circulation caused by cirrhosis of the liver, and it has been claimed by Hou (1958) that the thrombosis of the branches of the portal vein that is common in primary carcinoma of the liver was associated with oesophageal varices in 89% of his 200 cases of primary carcinoma of the liver in Chinese. The information available in the Liverpool cases was insufficient to confirm these figures but haemorrhage was common and was frequently the cause of death. In addition to alimentary bleeding from oesophageal varices haemorrhage was found also to occur into the peritoneal cavity or into the retroperitoneal tissues near the tumour. In a number of cases also, haemorrhage occurred at sites far removed from the diseased liver. Some of the haemorrhagic episodes, such as cerebral and cerebellar haemorrhages, were probably unrelated to the disease of the liver, but in some of the other cases, those with severe epistaxis, for example, it is possible that a tendency to bleed was a result of liver damage. The following type of haemorrhagic episode occurred in the Liverpool cases: alimentary 26, peritoneal 16, near tumour 6, distant 18.

DISCUSSION

The discovery of 111 cases in the Liverpool region suggested at first that the region had an unusually high incidence of primary malignant tumours of the liver. The incidence is usually expressed as a percentage of the total number of necropsies done during the relevant period, and the total number of necropsies was learned by asking all the pathologists in the region for the numbers of the necropsies in their institutions between 1 January 1947 and 30 June 1959. The information received indicated that the 88 necropsy cases of hepatic carcinoma had been yielded by 60,600 necropsies, an incidence of 0·145%, almost exactly the incidence for Europe generally (Berman, 1951). Such an incidence is somewhat less than the 0·2% found in Los Angeles and Chicago by Edmondson and Steiner (1954), and substantially less than the 0·72% incidence reported in Boston, Mass., by MacDonald (1957).

Although the incidence in the region as a whole was not unusually high, most of the cases were found in the vicinity of the ports of Liverpool and Birkenhead and the relatively large numbers of seafarers may give an indefinite hint of some aetiological relationship between seafaring and the disease.

The association with haemochromatosis, 7·95% of the necropsy cases, is remarkably similar to the

7·7% found by MacDonald in Boston, Mass. The lack of splenomegaly, too, in the majority of the Merseyside cases was similar to MacDonald's findings.

In general the Merseyside cases tended to be similar in type and behaviour to the cases studied in America and to a lesser extent to resemble the oriental and African type of case but the cases included such rare types as sarcoma of vascular endothelium, malignant mixed tumour of infancy, carcinoma following congenital cystic disease, and, possibly, primary malignant carcinoid tumour. The cases included two Chinese but no example of carcinoma complicating infestation with *Clonorcis sinensis* was recognized, and there was no case of carcinoma following accumulation of thorotrast in the liver.

Liver cell carcinoma was about four times more frequent than carcinoma of the intrahepatic ducts but, despite its remarkable tendency to permeate veins within the liver, appeared to be slightly less malignant in its behaviour than cholangiocarcinoma. It was an incidental finding, without metastases, in eight of the 88 necropsy cases, and when metastasis had occurred, lymph nodes were affected more commonly than organs involved by blood spread, perhaps because the veins invaded within the liver were eliminated from the circulation by thrombosis. In the 21 cases of carcinoma of intrahepatic bile ducts, the tumour was the main cause of death in nearly every case.

Calcification of primary carcinoma of liver cells may sometimes be so marked that the liver cannot be cut in the ordinary way (Lynch, 1958). But only microscopic foci of calcification in necrotic areas of tumour were seen in the Merseyside cases and such foci were infrequent and inconspicuous.

There was, unfortunately, insufficient information to indicate whether infective hepatitis or alcoholism had been important aetiologically in any of the cases.

I am indebted to Dr. J. S. Fulton, Director of the Liverpool Radium Institute, for permission to use the records of the Liverpool Cancer Control Organization, and to Dr. E. Mavis McConnell for much assistance in analysing the records and tracing cases.

All the pathologists in the Liverpool Region and Dr. G. Pantin, of the Isle of Man, have assisted by providing sections and by making their records available.

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The cytology of gastric mucosa

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SYNOPSIS Smears and sections were obtained from various labelled points on the mucosal surface of 25 freshly-excised stomachs, and the appearances were compared. Normal gastric mucosa, intestinal metaplasia, chronic gastritis, and carcinoma could generally be identified from the smears. In a few smears from the edge of peptic ulcers there were abnormal cells which might cause confusion with carcinoma. Conversely, tumour cells were seen which would be very hard to diagnose with certainty on cytological grounds.

The cytological examination of stomach contents has been used sporadically for the diagnosis of gastric carcinoma for over 50 years (Marini, 1909) and during the past decade has been the subject of numerous papers (see Rubin, 1956; Raskin, Kirsner, and Palmer 1958) and of two monographs (Henning and Witte, 1957; Schade, 1960). In spite of all that has been written, the pathologist examining gastric washings still encounters serious difficulties, especially in the separation of malignant cells from the altered cells which are sometimes found in chronic gastritis.

Heinkel, Witte, Landgraf, and Elster (1956) made a comparison between the findings of blind gastric biopsy and cytodiagnosis (the latter performed by the technique of Henning and Witte with a gastric swab). Only the varieties of gastritis were under investigation, and these authors described and illustrated abnormal cells which can be found in non-malignant conditions. It could not be claimed that the smears came from the areas of gastric mucosa taken for section, and in fact correlation was not very close. Although other workers in this field have no doubt made direct comparisons between smears and histological sections of the same area of gastric mucosa (Schade, 1958), they do not seem to have published their experiences. The present paper describes our findings in a direct comparison of smears and sections.

MATERIALS AND METHODS

The material consisted of 25 gastrectomy specimens. In nine cases a carcinoma was present, in eight cases the lesion was a benign gastric ulcer, and in eight cases the gastrectomy was performed for a duodenal ulcer alone. In one instance both a duodenal and gastric ulcer were present.

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Each specimen was taken directly to the laboratory, opened along the greater curvature, and then pinned on a cork board before fixation. From three to 12 different areas were selected for study.

Each area chosen was scraped with a scalpel and smears made on a glass slide with the scalpel blade. It was found that wooden or cardboard instruments were ineffective in removing surface epithelium. The smears were wet fixed in alcohol containing 3% acetic acid.

They were stained by Papanicolaou's method as well as by periodic-acid-Schiff (P.A.S.) or Mayer's mucicarmine (the latter was the better, as there was a tendency with P.A.S. for the cells to be obscured by brightly stained mucus). The areas from which the smears were obtained were labelled with numbered pins and the whole specimen fixed in 10% formal saline. After fixation, sections were taken of the numbered areas and stained with haematoxylin and eosin and with P.A.S.

In this way a series of histological sections of gastric mucosa was obtained together with smears of the epithelium from the same site. The material studied is classified in the Table.

RESULTS

The results were classified according to the histological appearances but without regard to the differences in distribution of the lesions in individual specimens.

NORMAL GASTRIC MUCOSA Normal gastric body mucosa was found in 16 sections from eight cases. Mild or focal superficial gastritis without abnormality of the superficial epithelium was found in 12 sections from nine cases. Thus a total of 28 sections (from 12 different cases) showed normal or almost normal gastric body mucosa.

FIG. 1

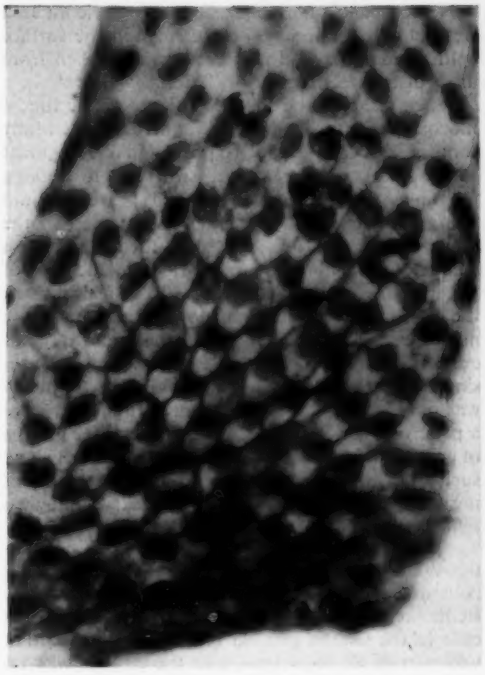

FIG. 2

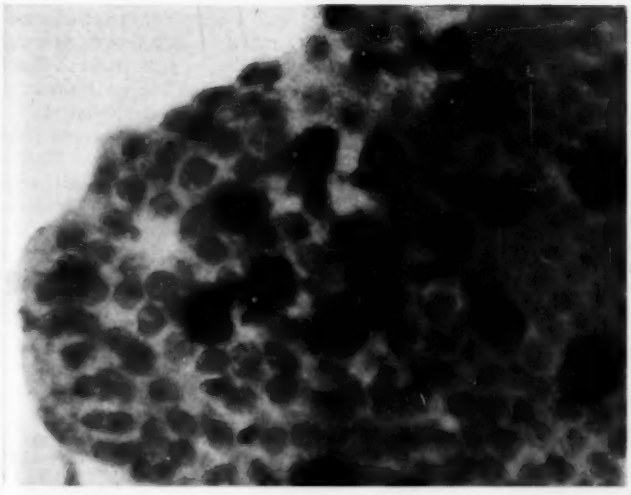

FIG. 3

FIG. 1. *Smear from normal gastric fundus. The sleeve-shaped structures are the linings of gastric pits. (Case G 101 area 6) (Papanicolaou × 150)*

FIG. 2. *Smear from normal gastric fundus (higher magnification of the same specimen as Fig. 1). The mucous vacuoles produce a honeycomb pattern. (Case G 101 area 6) (Papanicolaou × 700)*

FIG. 3. *Smear from duodenal mucosa, stained with P.A.S. The mucin in goblet cells is shown as darkly stained spheres overlying the nuclei. (Case G 51 area 5) (P.A.S. × 700)*

No completely normal sections of pyloric mucosa were obtained but very little change in the surface epithelium was noted in many sections taken from areas distant from any gross lesion.

When scraped, the superficial layer of the fundic mucosa peeled off in sheets of adherent cells. Many thousands of cells were often present in one fragment in the smears. Funnel-shaped structures resembling the fingers of mittens represented the epithelium lining the gastric pits (Fig. 1). When seen in the plane of the mucous vacuoles, the cells had a characteristic 'honeycomb' pattern made up of refractile hexagons (Fig. 2). This effect was lost when the cells were in small groups or were isolated. The nuclei were uniformly round or oval and did not exceed 11μ in diameter. Most of them contained a single nucleolus but some had two. The cytoplasm was only feebly stained with mucicarmine but gave a positive reaction with P.A.S. Although the groups of cells seen in smears from the pylorus did not show such a distinct architecture as those in the smears of normal gastric body mucosa, no real cytological difference was detected.

DUODENAL MUCOSA Thirteen sections from 11 cases consisted of or included duodenum. There was frequently some increase in lymphocytes and plasma cells in the lamina propria but in only one section was part of an ulcer seen, and the villi showed no gross distortion.

The smears from the duodenal mucosa showed sheets of cells with a similar but less marked 'honeycomb' pattern to those present in the stomach. Some of the sheets corresponded to the tips of villi. Goblet cells were well shown in smears stained for mucin (Fig. 3) and brush borders could be seen. The epithelial cell nuclei appeared identical to those of the gastric epithelium.

GASTRITIS A total of 79 sections from 24 cases showed gastritis. The morphological changes observed were similar to those described in gastritis by other workers (Magnus, 1937, 1946; Palmer, 1954; Joske, Finckh, and Wood, 1955). The severity of the lesions varied considerably even in individual sections, but although abnormalities of the surface epithelium were not always pronounced, the lamina propria always showed increased numbers of plasma cells and lymphocytes. Severe atrophic gastritis was found in six sections of gastric body mucosa. Sections of the pyloric mucosa and of the mucosa at the edge of the carcinomata and peptic ulcers almost always showed some degree of gastritis.

Active gastritis was frequently found in sections taken from the vicinity of the peptic ulcers and carcinomata. In such cases the surface epithelial cells were abnormal and neutrophil leucocytes were present in the surface epithelium and lamina propria. The surface epithelium was composed of flattened or cuboidal cells which had a darkly staining cytoplasm and a much reduced mucin content. The nuclei tended to be prominent and were sometimes enlarged with prominent nucleoli.

In smears, lesser degrees of superficial gastritis were not associated with any cytological changes in the epithelium. In more severe chronic inflammation the 'honeycomb' effect produced by the mucus-secreting layer was lost. Leucocytes (neutrophils and lymphocytes) were present in the sheets of mucosal cells and appeared to lie intracellularly. In most instances there was no particular change in the nuclei, but sometimes there was abnormal variation in size, heavier haematoxylin staining of the chromatin, and prominence of the nucleoli (Fig. 4).

In a few of the smears from areas of active gastritis, particularly at the edge of an ulcer, there was a considerable degree of anisonucleosis. In the example illustrated in Figs. 4 and 5, some of the nuclei measured as much as 15μ in their long axes, and they showed considerable overcrowding. The nucleoli were prominent, and in certain areas were stained red in the Papanicolaou preparations.

Smears obtained with the scalpel included whatever leucocytes were present in the lamina propria. In some cases plasma cells were numerous. These cells have not in our experience been present in gastric washings (although 'pseudoplasmacytes'—pyknotic forms of epithelial cells sometimes containing hyaline droplets—may be abundant). Plasma cells were not seen in or among the epithelial cells in sections, and it appears that they do not penetrate the mucosa.

In one case of gastric ulcer the smears contained collections of what appeared to be cells from the gastric glands. The corresponding section showed a group of gastric gland cells apparently extruded on to the surface (Figs. 6 and 7). Although this appearance is sometimes seen in histological sections, the glandular cells do not seem to be recognizable in saline washings from the stomach. They have, however, been seen in gastric swab preparations by Henning and Witte (1957).

No case of Addisonian pernicious anaemia was included in this series.

TABLE

HISTOLOGICAL MATERIAL

Site	*No. of Sections*
Stomach	93
Duodenum only	6
Edge of benign peptic ulcer of stomach	15
Carcinoma of stomach	21
Total	135

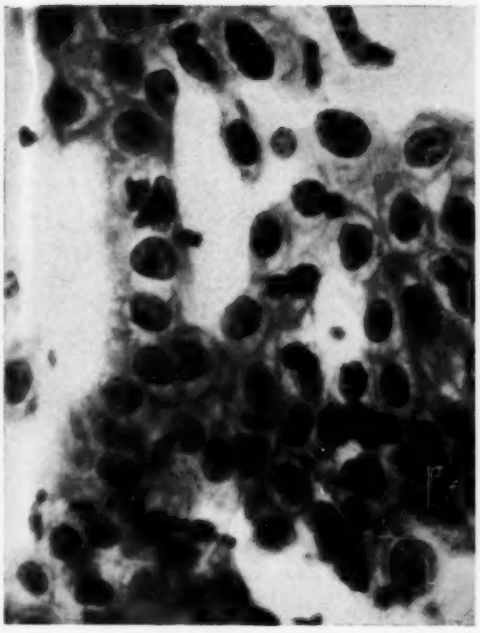

FIG. 4. *Smear from the edge of a simple gastric ulcer. The mucous vacuoles are lost, and the nuclei show variations in size, hyperchromasia, and prominent nucleoli.*
(Case G 97 area 6) *(Papanicolaou × 700)*

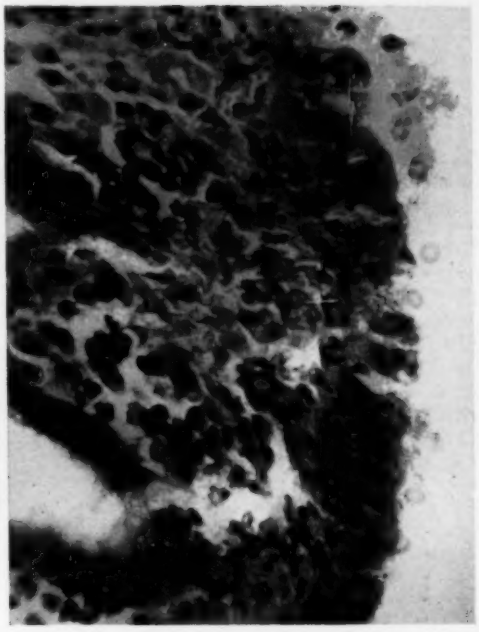

FIG. 5. *Section showing abnormal surface epithelium in an area of active gastritis at the edge of a benign peptic ulcer. Same area as Fig. 4.*
(Case G 97 area 6) *(Haematoxylin and eosin × 310)*

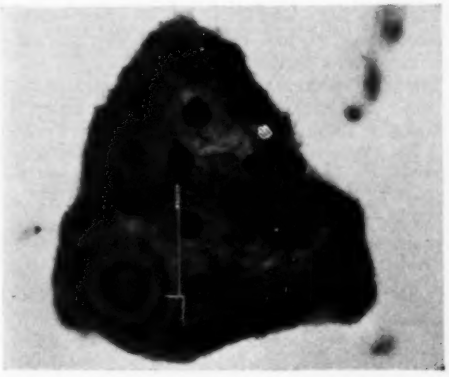

FIG. 6

FIG. 6. *Smear from an area of chronic gastritis, showing a collection of cells from a gastric gland.*
(Case G 18 area 10) *(Papanicolaou × 700)*

FIG. 7. *Section showing fundic gland cells on surface of gastric mucosa. Chief and parietal cells are present. Same area as Fig. 6.*
(Case G 18 area 10) *(Haematoxylin and eosin × 375)*

FIG. 7

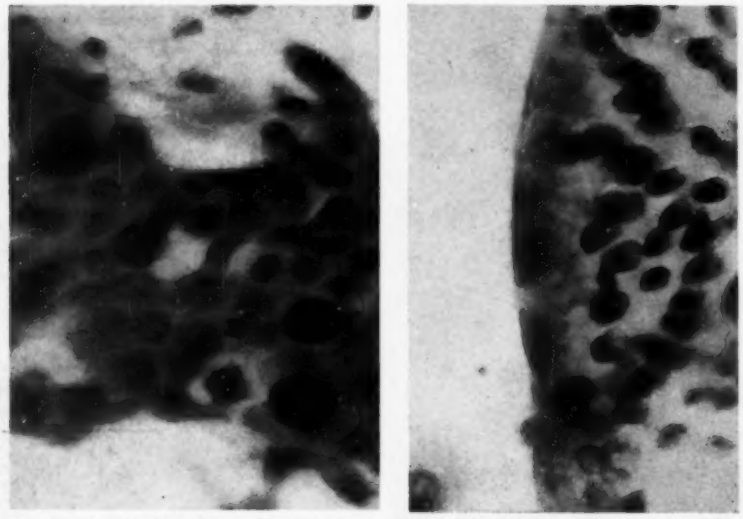

FIG. 8. *Smears from areas of intestinal metaplasia of the stomach. Left, P.A.S. preparation showing 'goblets', similar to those in Fig. 3. (Case G 116 area 3)* (× *700*) *Right, fragment showing cuticular border. (Case 38 area 5) (Mucicarmine* × *700)*

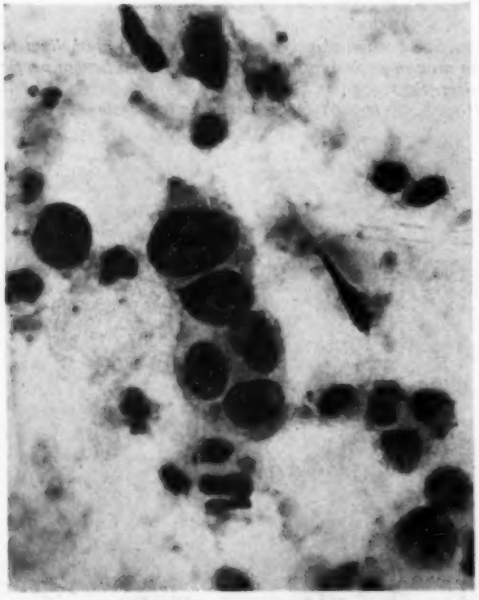

FIG. 9. *Smear from gastric carinoma. These nuclei show the characteristic enlargement, hyperchromasia, variation in size, and prominence of nucleoli, exceeding the changes found in gastritis. (Case G 117 area 2) (Papanicolaou* × *700)*

INTESTINAL METAPLASIA Areas of intestinal metaplasia were found in one or more sections from 16 cases. A carcinoma of the stomach was present in seven cases, a duodenal ulcer was present in six cases, and a gastric ulcer was present in three cases. In four cases of carcinoma large areas of mucosa had undergone metaplasia, but in the others the change was slight and focal. Goblet cells, a striated epithelial border, and Paneth cells were present in the metaplastic epithelium.

Intestinal metaplasia was also recognizable in the smears. The refractile 'honeycomb' of vacuoles was reduced or absent[1], but the goblet cells were present and were particularly prominent in smears stained with mucicarmine or P.A.S. (Fig. 8). In some fragments the cuticular or brush borders were well shown (Fig. 8). The nuclei showed no characteristic features.

GASTRIC CARCINOMA The cases included a diffuse anaplastic carcinoma (linitis plastica), a signet ring cell (mucus-secreting) adenocarcinoma, five cases of adenocarcinoma which showed varying degrees of glandular differentiation; also an early, poorly differentiated adenocarcinoma which was confined to the mucosa at the edge of a benign peptic ulcer.

[1]Probably owing to gastritis: in gastric washings from cases of pernicious anaemia, pieces showing intestinal metaplasia may have well-preserved refractile vacuoles.

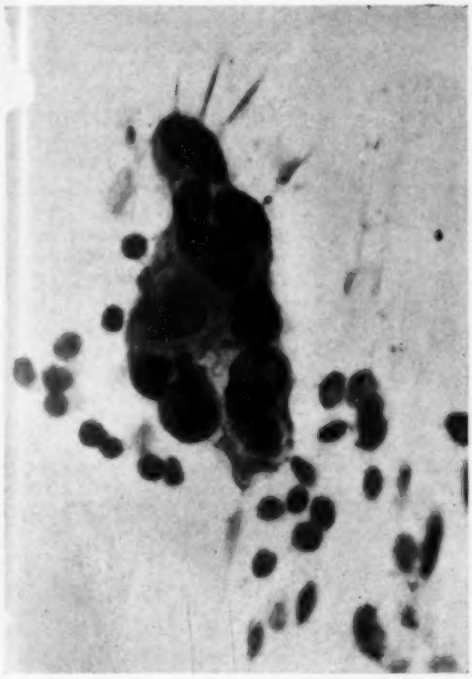

FIG. 10

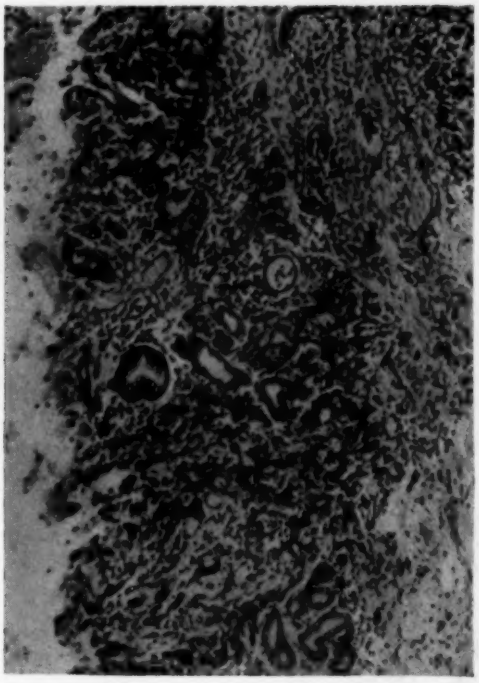

FIG. 11

G. 10. *Smear from an unsuspected microscopical carcinoma at the edge of a peptic ulcer.* (*Case G 22 area 3*) *Papanicolaou* ×*700*)

FIG. 11. *Section of small, unsuspected, poorly differentiated gastric adenocarcinoma found at the edge of a peptic ulcer. The growth was confined to the mucosa. Same area as Fig. 10.* (*Case G 22 area 3*) (*Haematoxylin and eosin* × *140*)

The tumour cells found in gastric scrapings in this series have fallen into three main groups, which correspond to the categories of cells found in gastric washings. First (Fig. 9) were cells occurring either separately or in small collections, with marked variation in size and shape of the nuclei, a high ratio of nucleus to cytoplasm, hyperchromasia, and enlargement of nucleoli. Such cells were not difficult to identify and nothing similar was found in chronic gastritis. If coherent pieces of tumour were scraped off as well, they had ill-defined edges and a very irregular and overcrowded appearance. The tendency of individual cells to come loose from the main mass was most characteristic and was not observed in benign conditions.

In one case of gastric ulcer which appeared benign o the naked eye, the scrapings showed cells of this ype and the sections revealed a small early carcinoma at the edge of the ulcer (Figs. 10 and 11).

The second variety of tumour cell shown in the series was the signet ring cell. This was present in the scrapings in only one case (Fig. 12). Except for a considerable variation in size these cells showed no obvious malignant characteristics. The cells measured 15-35 μ across, and were mainly occupied by a foamy cytoplasm staining positively with P.A.S. and mucicarmine. The nuclei (generally one per cell, but sometimes two or three) lay eccentrically, measured 8-10 μ across, and showed distortions and foldings like those of macrophages. Very few of them were hyperchromatic. Unlike macrophages, they showed well-defined nucleoli.

The third type of tumour cells (encountered in three cases) seemed to show greater mutual adhesiveness, so that they came off in sheets. In one case, where loose malignant cells were also found, the large size of the nuclei made the diagnosis easy. Some nuclei measured as much as 20 μ in one dia-

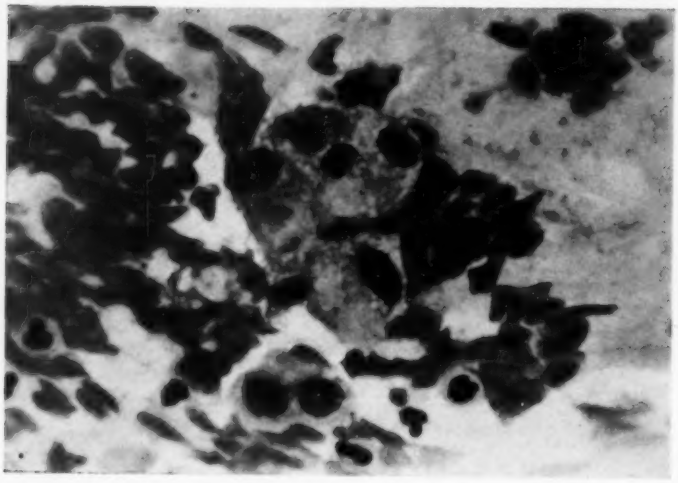

FIG. 12. *Smear from a signet-ring carcinoma. The tumour cells have foamy cytoplasm and eccentric nuclei larger than those of macrophages.* (*Case G 33 area 1*) (*Papanicolaou* × *700*)

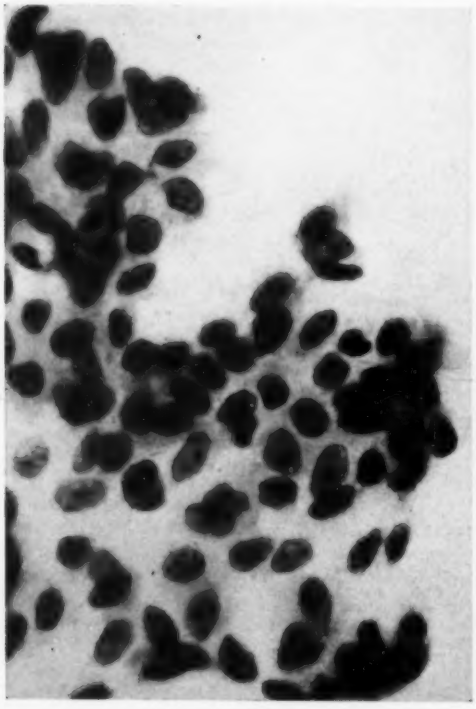

FIG. 13. *Smear from a gastric carcinoma. In this case the cells came off in sheets with no sign of polarity, but the cells showed no greater cytological abnormalities than are sometimes found in chronic gastritis.*
(*Case G 116 area 1*) (*Papanicolaou* × *700*)

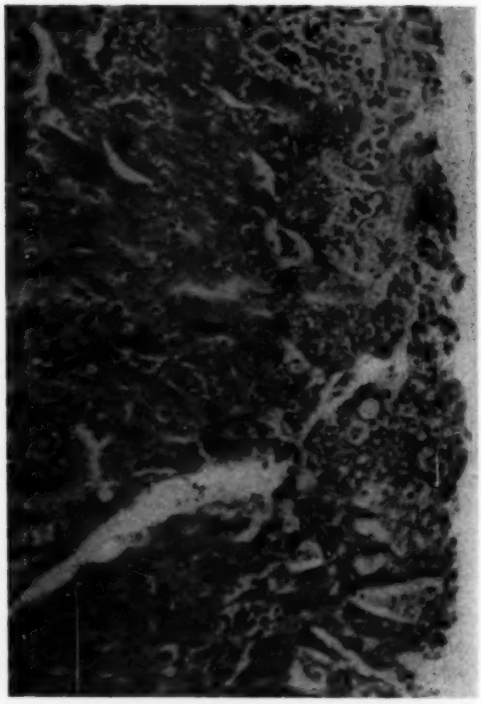

FIG. 14. *Section showing the surface of a poorly differentiated adenocarcinoma of stomach. Same area as Fig. 13.*
(*Case G 116 area 1*) (*Haematoxylin and eosin* × *140*)

meter. In another case, only an occasional nucleus 17 μ long was outside the usual range for benign cells in gastritis. In the third case, the cells scraped from the tumour surface were, in our opinion, not individually distinguishable as malignant. Moreover, the degree of architectural disorder was not greater than was sometimes found in active gastritis; their main peculiarity was a tendency to separate as spindle cells. Small groups of these cells occurring in gastric washings would certainly be passed without suspicion (Figs. 13 and 14).

In one case of linitis plastica none of the smears showed any recognizable malignant cells. As far as could be seen from the corresponding sections, the tumour cells did not reach the surface of the mucosa.

DISCUSSION AND CONCLUSION

The morphological appearances of the cells obtained by scraping the mucous membrane of the excised stomach correspond closely with those of cells found in gastric washings. The results of this study seem therefore to be directly relevant to gastric 'cytodiagnosis'. If enough cells of gastric origin can be found, it is possible to distinguish the following conditions:

NORMAL This is recognized by the regular architecture of the fragments, the 'honeycomb' of the mucus-secreting layer, and the absence of cellular infiltration. (This appearance has also been observed in gastric washings from cases of Addisonian pernicious anaemia.)

CHRONIC GASTRITIS The 'honeycomb' appearance is lost, the nuclei become variable, more darkly staining, and sometimes enlarged, and there is leucocytic infiltration. At times the appearances are indistinguishable from well-differentiated carcinoma.

INTESTINAL METAPLASIA This can be diagnosed from the finding of goblet cells and of cuticular or brush borders. Large fragments may show the actual architecture of villi. In gastric washings this finding indicates atrophic gastritis or gastric atrophy, and has been observed in pernicious anaemia.

CARCINOMA In most cases malignant cells are easily recognized by their large, irregular hyperchromatic nuclei with prominent nucleoli, their tendency either to separate from one another or to form crowded aggregates showing anisonucleosis, and sometimes by numerous mitoses. The signet ring type is also readily recognizable. However, no two malignant tumours are exactly alike, and the variations include forms in which the cells are not distinctive. In this very small series there are two examples in which the isolated cells could not be distinguished with certainty from types of cells found in the neighbourhood of a benign peptic ulcer.

This study therefore emphasizes the fact that in trying to diagnose carcinoma of the stomach from cytological evidence, we are working very near to the margin of what is humanly possible. Rubin and Benditt (1955), Raskin *et al.* (1958), and Schade (1960) have claimed that carcinoma can be diagnosed in this way in over 90% of cases, and consider that a negative finding is of actual diagnostic value in helping to exclude malignancy. Most other workers have had a higher proportion of 'false negative' findings. The present series illustrates two simple causes of failure: first, the malignant cells may not reach the lumen, and second, they may not be sufficiently distinctive morphologically for a definite diagnosis. False positive reports can only be avoided if the examiner is familiar with the aberrations which may occur in active gastritis, and direct comparisons such as ours are valuable in obtaining experience of these changes.

We are grateful to Dr. A. H. T. Robb-Smith for his advice and for reading the manuscript; also to our surgical colleagues who have kindly supplied us with material, and to Mrs. D. Jackson for photographs of the histological sections.

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J. clin. Path. (1961), **14**, 140.

The adhesiveness of native platelets and its prevention

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SYNOPSIS The following methods were used to measure the adhesion to various surfaces of platelets in whole blood or plasma: 1, two measurements gave an estimate *in vivo* of platelet adhesion to cut human capillaries; 2, platelets adhering to damaged cells *in vitro* were counted directly; 3, a highly reproducible method for estimating platelet adhesion to glass was devised; 4, the manner in which platelets adhere to each other (aggregation) was also studied.

Platelet adhesion to all these surfaces was found to be dependent upon calcium and independent of all clotting factors except that platelet aggregation is probably dependent upon thrombin. A number of drugs—mostly antimalarials, antihistaminics, and local anaesthetics—in suitable concentration inhibited adhesion. They probably form a fixed, orientated layer on glass and possibly on cells and make these surfaces unattractive to a platelet. They also stick reversibly to live cells (including platelets) altering their permeability, and they may make platelets less adhesive. Consequently the possibility of using antiadhesive drugs therapeutically to inhibit thrombus formation was considered.

An arterial thrombus may be composed mainly of fibrin, or as seems more possible, of a mass of platelets. It is, however, most probable that an essential early link in the chain of events during the formation of the thrombus must be the adhesion of a few platelets to the vessel wall, and it is probable that the precipitating event is a local change in the nature of the endothelial surface. Most work on the adhesiveness of platelets has been carried out on plasma or blood stabilized by citration or heparinization and using a glass surface. In the present study it was decided to use moving native whole blood wherever possible since using anticoagulants might obscure the phenomenon to be investigated. The object was threefold: 1, to devise quantitative methods to study platelet adhesiveness to 'damaged' cells, to artificial surfaces such as glass, and to each other; 2, to elucidate the mechanism of adhesion; and 3, to investigate the possibility of preventing this phenomenon.

We have succeeded in devising some quantitative methods, but we have learnt only a little about some aspects of the mechanism involved, and have found a number of drugs which, in high concentration, interfere with platelet adhesion *in vivo* and *in vitro*. Some of this work has been previously reported and its relation to thrombosis considered (O'Brien, 1960).

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MATERIALS

NATIVE PLATELET-RICH PLASMA Whole blood was centrifuged in siliconed tubes at 1,000 r.p.m. for 10 minutes, and the supernatant collected.

NATIVE PLATELET-POOR PLASMA Blood cooled to 4°C. was centrifuged at 17,000 r.p.m. (35,000 *g*) for 20 minutes.

WASHED PLATELETS The platelets in citrated platelet-rich plasma were washed five times in normal saline.

PLATELET FRAGMENTS The platelets in citrated plasma or washed platelets were ruptured by ultrasonic agitation.

AMNION CELLS Confluent sheets of human amnion cells were grown on glass slides using Lane and Marshall's method (1957) but with added human serum. The drugs were used in the concentrations stated, diluted in normal saline.

METHODS

PLATELET ADHESION TO DAMAGED CELLS Two methods were used to measure this: 1, The Ivy bleeding time, as modified by O'Brien (1951), and 2, the method of Borchgrevink (1960) as follows.

A sphygmonmanometer cuff on the upper arm was inflated to 40 mm. Hg, then a standardized, very superficial cut, 10 mm. long, was made on the volar aspect of

the forearm. Two platelet counts were taken from the first free flowing drops of capillary blood: thereafter the cut was blotted as in the Ivy technique to determine the bleeding time. The mean of these platelet counts was then expressed as a percentage of the venous platelet count. Normally the capillary platelet count is about 30% lower than the venous count. This figure will be called the percentage of platelets adhering, since it must be due to the platelets sticking to the cut capillaries (and the skin wound) to form a platelet plug which will eventually stop the bleeding.

The drugs to be tested for anti-adhesive properties were injected intradermally to make a bleb 10 mm. wide and a cut was subsequently made across this bleb. The percentage of platelets adhering and the bleeding time were compared with identical cuts made at the same time over sites where saline had been injected. It was found that in all the situations studied there was a rough inverse linear relation between the length of the bleeding time and the percentage of platelets adhering. A low percentage of adhering platelets was followed by a long bleeding time and *vice versa*. Thus, in later studies when drugs were injected, only the bleeding times were measured.

PLATELET ADHESION TO AMNION CELLS Confluent sheets of human amnion cells on glass were 'damaged' by heating to 56°C. for 30 minutes, and small pieces of the glass were broken off and stuck into microscope slides. Fresh whole blood collected by venepuncture was trickled for a predetermined time, usually 15 seconds, over the mounted small portion of cell-covered glass. The whole slide was then inverted over a pot quite full of formol saline so that the slide was wetted and the red cells fell to the bottom. The slide was then washed and stained with brilliant cresyl blue. The platelets in 10 to 20 random microscope fields were counted under a 1/12 objective, and the mean number per field calculated. Other syringes were filled with blood from the same venepuncture and solutions of the drugs under investigation sucked into the syringes, mixed, and the treated blood was then trickled over other portions of the same sheet of amnion cells. The results for each substance added are reported as the average number of platelets adhering to the cells in the presence of the substance, expressed as a percentage of the number adhering when saline was added to the blood; thus 50% adhesion indicates that only half as many platelets adhered in the presence of the substance as when saline was added.

PLATELET ADHESION TO GLASS This was studied using a glass filter unit and microscopically.

Glass filter unit Blood was collected through a wide-bore siliconed needle into a 40 cm. length of plastic tubing of 5 mm. diameter, and the tubing divided between clamps into halves to allow for replicate observations. One end of one of the halves was connected to a 2 ml. syringe containing liquid paraffin and the other end of the tube connected to a 'filter unit'. The filter unit of glass tubing of standard dimensions (Fig. 1) was filled with glass beads held between two sheets of nylon gauze. As soon as the blood-filled plastic tube was connected, a machine forced the syringe plunger down to a constant speed and

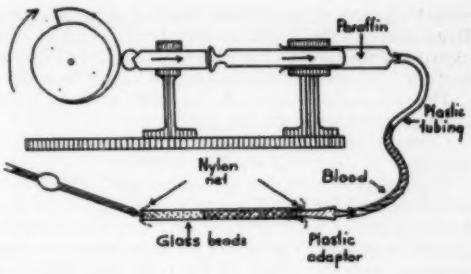


FIG. 1. *Apparatus for measuring adhesiveness of native platelets to glass.*
Transit time, 15 sec., pore size 60μ, no. of beads 19,000, each 0·47 mm. diameter, total surface area 14,000 mm.²

the blood was pushed through the filter; the leading edge of the blood took 15 seconds to traverse the filter. (Hugues, 1959, reports that the speed of blood flowing in an arteriole is 50 mm./sec. and is equivalent to traversing 7 cm. in 14 seconds.) An initial platelet count of at least 200 platelets, using Feissly and Ludin's method (1949), was made on the blood in the plastic tube before it passed through the filter; a second count was made on the first drop issuing from the filter, and a third count taken 15 seconds later when about 1 ml. of blood had passed through the filter. Similar procedures were carried out on the second half of the original plastic tube and the results averaged. The difference between the initial and second count, expressed as a percentage of the initial count, is a measure of the number of platelets that stick in the leading front of the blood passing over a large, dry glass surface of known area. The difference between the initial and the third counts, expressed as a percentage of the initial count, is a measure of the number of platelets that stick to glass which has been in contact with the blood for 15 seconds. The first percentage will be called 'the percentage of platelets adhering initially' and the second 'the percentage of platelets adhering later'. The percentage of platelets adhering was found to be independent of the initial platelet count within wide limits. If additions were to be made to the blood, the blood was collected into siliconed tubes containing the addition and mixed, and the plastic tube was filled from this mixture. The figures reported are the means of four or more experiments with each substance added.

Microscopic observation Native platelet-rich plasma, with or without additions, was placed on a glass slide under a coverslip, and adhesion of the platelets to the glass was studied while the coverslip was rocked, using the 1/6 objective and phase illumination.

ADHESION OF PLATELETS ONE TO ANOTHER (AGGREGATION) Platelet-rich native plasma, 0·3 ml., with or without additions was pipetted into glass tubes 7 mm. in diameter and agitated in a water-bath at 37°C. They were watched through a strong hand lens with powerful cross illumination. Four stages could usually be observed and timed,

namely, a smooth suspension, the first signs of granularity, coarse 'snow flakes', or complete aggregation with clearing of the surrounding plasma when all the platelets had stuck to each other in clumps, and fibrin formation.

The microscopic method, outlined under 'Platelet Adhesion to Glass', was also used.

Coagulation tests were carried out essentially as described by Biggs and Macfarlane (1957).

The sedimentation rate was determined using blood anticoagulated by E.D.T.A., to which was added one tenth volume of saline or other addition. The Wintrobe method was used. The results for each substance were expressed as a percentage of the result obtained when saline only was added, and are the average of several experiments using different bloods with moderately raised sedimentation rates.

CLOT RETRACTION The clot retraction test was carried out by adding 0·9 ml. of blood to 0·1 ml. of the solution under investigation in a clotting tube in the water bath at 37°C. The clot was gently loosened from the wall of the tube, and, one hour later, the results on inspection were recorded as normal, poor, or absent.

RESULTS

REPRODUCIBILITY OF RESULTS The poor reproducibility of the bleeding times estimated by the Ivy technique has been previously reported (O'Brien, 1951). The Borchgrevink capillary platelet count showed some variation (the figures reported being the mean of 10 to 20 observations) but the bleeding times were reasonably reproducible. The active drugs at the concentration used usually grossly lengthened the bleeding times, so replicate bleeding times were necessary. Testing the adhesion of platelets to heated amnion cell sheets is somewhat erratic and at best only semi-quantitative. For each substance the test was repeated sufficiently often to give complete confidence in the trend, if not in its exact quantitation. Occasional inexplicably erratic results were recorded with the glass filter unit but on the whole this test was found to be remarkably reproducible. The microscopic inspection of adhesion permitted differentiation with certainty only on an all-or-none basis. Platelet aggregation in tubes was highly reproducible as an all-or-none phenomenon.

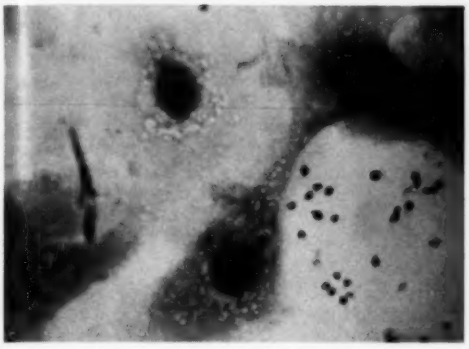
ADHESIVENESS OF PLATELETS IN SOME HAEMORRHAGIC DISORDERS The following diseases were examined using the glass filter units (the number of patients examined is put in brackets). Hageman trait, severe (2), haemophilia, moderate to severe (2), Christmas disease, severe (1), Dindevan-treated patients with a prothrombin time of 35 to 60 sec. (3), congenital afibrinogenaemia (1). One patient with the Hageman trait gave one aberrant platelet count but the percentage of platelets adhering later was normal (58%). The other case was normal (55% and 60%). The results of all the other patients listed above were strictly within the normal range.

Three cases of von Willebrand's disease were studied. Two gave strictly normal results in the glass filter units. The third, a boy with difficult veins, was examined three times; twice he gave normal results and once they were abnormal. The bleeding time in all these coagulation defects, except in von Willebrand's disease, is said to be normal, and was not checked in this study: thus probably platelet adhesion to a damaged vessel wall is normal. In a larger series of coagulation defects the adhesion of platelets in citrated blood to glass was reported as normal by Hellem (1960). From this report and the present results it may be concluded that very probably none of the coagulation factors studied is concerned with platelet adhesion to glass or to damaged endothelial cells.

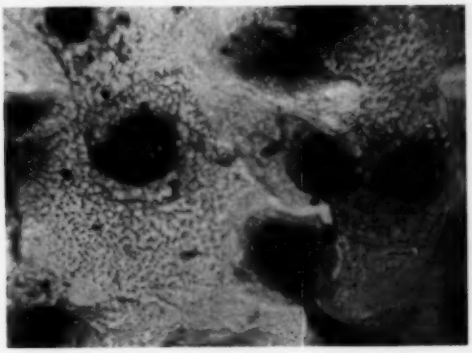
PLATELET ADHESION TO DAMAGED CELLS Many drugs were next investigated to see if they inhibited the adhesion of platelets under the various test situations in an attempt to understand the underlying mechanism(s).

Borchgrevink method All drugs injected were used at a concentration 10 times that indicated in the second column of Table I. When quinine, benadryl, and cocaine were injected, the percentage of adhesive platelets was lower than when saline was injected. This indicated that fewer platelets stuck to the cut vessels and the bleeding times were correspondingly grossly prolonged. The bleeding times after injecting most of the drugs with anti-adhesive properties in glass (vide infra) were grossly prolonged. It is remarkable that procaine and chloryclaizine gave normal bleeding times. Some drugs caused tiny areas of necrosis, but the necrogenic effect was independent of the effect on the bleeding time. Some drugs produced erythema and/or a wheal; this was thought to be irrelevant, since histamine injected produced the same clinical effect but the percentage of platelets adhering and the bleeding times were normal. If drugs were injected in strengths 10 times weaker, *e.g.*, 2·5 mg. quinine per ml. saline, then little or no lengthening of the bleeding time was observed.

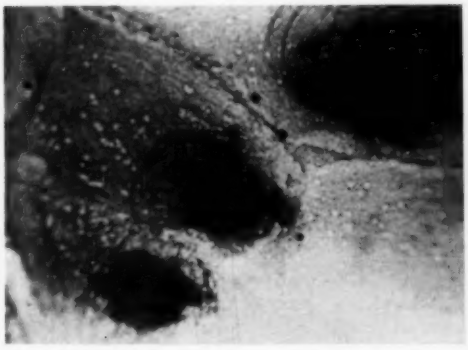
Amnion cells When blood was trickled over live amnion cells no platelets adhered to the cells (Fig. 2), although they adhered to any uncovered glass. When blood was trickled over confluent heat-damaged cells for five seconds before fixation, some platelets had already stuck. After 15 seconds many platelets had stuck but fibrin was not formed for a further 15 or more seconds. (Exposure to blood for 15 sec. was routine.)



A Blood trickled for 30 seconds over untreated cells: no platelets have stuck to the cells, but many platelets have stuck to the glass where the cells were non-confluent.



B Blood trickled for five seconds over heated cells: a few platelets have already stuck (an example of adhesion long before clotting).



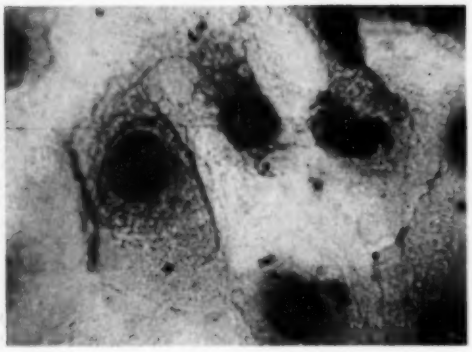
C Benadryl-treated blood trickled for 15 seconds over heated cells: a few platelets have stuck.

D Untreated blood trickled for 15 seconds over heated cells: many platelets have stuck.

FIG. 2

Some substances that had inhibited adhesion to glass (vide infra) were mixed with blood in the same concentration as that used in the filter units and the blood was then trickled over the damaged cells. It will be seen from Fig. 2 and Table I that in the presence of all but two of the active drugs tested, markedly fewer platelets adhered to the amnion cells. Thrombodym and reserpine did not interfere with platelet adhesion in this test system. It is remarkable that few platelets in heparinized blood stuck to the amnion cells.

ADHESIVENESS OF PLATELETS AND WHITE CELLS TO GLASS The substances listed in Tables I and II were tested by adding them to native whole blood and then passing the mixture through the glass filter units. The concentration (recorded in mg. of drug per 1 ml. of blood) is that which produced a marked effect. A concentration one tenth as strong usually produced a moderate but less marked effect, and usually a concentration 10 times stronger had little greater effect, or haemolysed the red cells or platelets. On every occasion a run was made blood with added saline was examined as a control, but individual controls have been omitted and an overall mean figure is given. It will be seen that the addition to the blood of most of the drugs listed in Table I caused a moderate initial decrease in the number of platelets that stick compared with the saline control, while later virtually none stick. It was also shown, at least with Benadryl, that while many platelets stick initially, few platelets had been lost from blood



E Benadryl-treated blood trickled for 30 seconds over heated cells: appearance similar to C.

F Untreated blood trickled for 30 seconds over heated cells: fibrin strands have formed in the direction of blood flow (horizontally in this picture) and many platelets in clumps and undergoing viscous metamorphosis have stuck everywhere.

G Benadryl-treated blood trickled for 45 seconds over heated cells: no platelets have stuck to the cells, but the blood has clotted and platelets stick to fibrin strands only (an example of clotting in the complete absence of platelet adhesion to the surface).

H Untreated blood trickled for 45 seconds over heated cells: much fibrin, platelet clumps, and viscous metamorphosis.

FIG. 2

collected about three seconds after the first drop issued from the end of the filter units. It seems that in the presence of Benadryl there must be a very rapid change in the glass-blood interface which renders it less attractive to a platelet. Since the other tests also showed that these drugs prevented platelet adhesion, it is probable that the 'percentage of platelets adhering later' is the more revealing figure, and that the percentage adhering initially does not reflect the overall picture. The positively charged detergents CTAB and cetyl pyridinum bromide inhibited adhesion while neutral (Tween 80) and negatively charged (Empicol L.Z.) detergents had much less effect. At high concentration salicylates markedly decrease adhesiveness, while at a concentration of 1 mg./ml. blood there is still some definite inhibition. The adhesion of platelets to glass was prevented by citration, but heparin had a negligible effect. Table II lists a number of substances that had little or no effect on platelet adhesiveness, and adhesiveness was not reduced at 2°C. The effect of reserpine was difficult to isolate from the effect of its solvent, which was also inhibitory.

The white cell count of native whole blood passed through the glass filter did not alter. It may be

TABLE I

ANTI-ADHESIVE AND SOME OTHER EFFECTS OF DRUGS ADDED TO BLOOD

Added	*Concentration in Blood (mg./ml.)*	*Borchgrevink Bleeding Time*[1]		*% Platelets Adhering to Heat-damaged Amnion Cells*	*Glass Filter Units*		*Platelet Aggregation*	*Clot Retraction*	*Sphering of Platelets*	*Red Cell Sedimentation Rate as % of Saline Control*	*Nature of Glass Surface Coated with Drug*
		% Platelets Adhering	*Bleeding Time (min.)*		*% Platelets Adhering Initially*	*% Platelets Adhering Later*					
	90·0	32	3·5	100	60	75	Normal	Normal	Absent	100	Hydrophilic
HCl	2·5	5	>15	40	32	−5[3]	Absent	Absent	Complete sphering	12	Markedly hydrophobic
HCl	1·0				34	17	,,	,,		30	—
uine SO_4	1·0		12·0		27	3	,,	,,	Moderate sphering	60	Markedly hydrophobic
ne SO_4	1·0		6·7		20	−6	,,	Poor	,, ,,	50	,, ,,
e HCl	0·3		8·0		26	15	,,	,,	,, ,,	50	,, ,,
l	1·0	21	11·0	40	53	−9	Absent	Absent	Complete sphering	10	Markedly hydrophobic
n	1·0		10·7		20	−6		Poor	,, ,,	22	,, ,,
an	2·5		14·0		37	12	,,	Absent	,, ,,	0	Moderately hydrophobic
izine HCl	0·1		2·7N		50	8	,,	,,	,, ,,	3	Markedly hydrophobic
HCl	3·0	12	11·4	27	36	8	Absent	Absent	Complete sphering	38	Markedly hydrophobic
ine HCl	1·0		15·0N		18	22	,,	Poor	,, ,,	4	,, ,,
ine HCl	10·0		11·0		50	12	Poor	,,		15	,, ,,
HCl	5·0		4·0	30	25	−3	Normal	Normal	Moderate sphering	90	Moderately hydrophobic
amide HCl	1·0		4·2		44	24	Poor	,,	Absent	90	,, ,,
mazine HCl	1·0		5·5N		31	19	Absent	Absent	Complete sphering	3	Markedly hydrophobic
azine citrate	5·0		10·0		36	17	,,	,,	Absent	100	Moderately hydrophobic
e	2·5		7·0N	110	42	13	,,		,,	100	,, ,,
odym	2·25			100	26	24	,,	Poor	,,	90	Hydrophilic
e	10·0		5·0[4]	40	16	14	Absent	No Clot	Complete sphering	0	Moderately hydrophobic
	1·0				24	12	,,	,, ,,	,, ,,	0	Markedly hydrophobic
	0·1			30	42	50	,,	,, ,,	Absent	100	Hydrophilic
	380		13·0		28	−3	,,	,, ,,	,,	100	,,

s test substances injected were × 10 the concentration in column 2.
ates that injecting these substances usually produced an area of necrosis 2 mm. in diameter.
s sign indicates that this treated platelet count was higher than the initial count.
e injected at a concentration of 10 mg./ml.

concluded that under these conditions white cells do not stick to glass.

PLATELET AGGREGATION All the drugs listed in Table I, except procaine, prevented aggregation. Positively charged detergents, procaine amide, and lignocaine had little effect. A strong salicylate solution prevented aggregation and clotting, but at a concentration of 1 mg./ml. aggregation was normal. Aggregation was also prevented by citrate and by a strong heparin but by no other drugs tested. This suggests that platelets cannot aggregate in blood that does not clot.

TABLE II

ADHESIVENESS OF PLATELETS TO GLASS

Heparin	0·3[1]
Liquoid	0·01
Protamine SO_4	10·0
Dextran SO_4	2·5
Dextran (Mol. Wt.180,000)	30·0
Tween 80	1·0
Empicol L.Z.	0·1
Uranyl SO_4	1·0
Digitonin	sat. solution 100
Saligenin	10·0
Mephenesin	3·0
Conessine	0·3
Tubocurarine	50·0

[1]Concentrations in mg./ml. blood

CLOT RETRACTION In the presence of most of the active drugs the clot did not retract (Table I). Clot retraction was poor or absent when the positively charged detergents were added. It is remarkable that procaine and sodium salicylate, 1 mg./ml. blood, had no effect, *i.e.*, clot retraction and platelet aggregation were normal.

BLOOD COAGULATION The following tests were carried out using most substances: the clotting time in glass tubes, the one- and two-stage prothrombin times, the prothrombin consumption, and the thrombin generation test, the last using native whole blood and citrated blood and platelet-rich and platelet-poor plasma. When the drugs listed in Table I were added to blood in the concentra-

tions indicated clotting was normal in every test.

The following minor exceptions were noted. Cocaine and procaine usually delayed clotting. Cocaine, quinine, and Benadryl usually caused slightly more thrombin to be formed than the saline control, whether platelets or a cephalin substitute was used. Weak CTAB caused a long one-stage prothrombin time, but the two-stage test was normal. CTAB, 1 mg./ml., prevented clotting. The clotting time and thrombin generation with added salicylate (1·0 mg./ml. blood) was poor, and 10 mg./ml. prevented clotting and made the blood slightly acid.

PLATELET MODELS The following models were studied: fresh platelets from citrated blood washed five times in saline; platelets from 14-day-old A.C.D. bank blood; platelet fragments; cephalin particles; and glass particles 2μ in diameter. All these models suspended in native plasma did not adhere to glass at all or markedly worse than fresh platelets. They did not aggregate and produced poor or absent clot retraction. It is concluded that fresh intact platelets are essential for the production of these phenomena.

PLATELET ADHESION IN PRESENCE AND ABSENCE OF RED CELLS Hellem (1960) claims that platelets stick to glass only if red cells are present. His experiments with citrated blood and plasma were repeated using the present method, and native blood and native platelet-rich plasma prepared from the same venepuncture were examined as well. The results of three such experiments are summarized in Table III.

TABLE III

PLATELET ADHESION TO GLASS IN PRESENCE AND ABSENCE OF RED CELLS

	Percentage of Platelets Adhering Initially	*Percentage of Platelets Adhering Later*
Native blood	52	48
Native platelet-rich plasma	−4	23
Citrated blood	37	14
Citrated platelet-rich plasma	−23	−4
Native platelet-rich plasma + benadryl, 1 mg./ml.	−15	−7

A minus figure indicates that the percentage of platelets issuing from the filter is higher than the pre-treatment count.

In blood and in plasma citration causes fewer platelets to stick. Whether the plasma is citrated or not, fewer platelets stick in the absence of red cells. However, some platelets (23%) do stick in native plasma in the absence of red cells and their adhesion is inhibited by Benadryl.

RESULTS *in vivo* During a control period of four or more days four volunteers were studied daily using the Ivy bleeding time test and the glass filter units. These volunteers then ingested sufficient sodium salicylate to raise the blood salicylic acid level to 20 to 30 mg.% and the tests were repeated. No change was found.

HOW DO THE ANTI-ADHESIVE DRUGS WORK?

The following experiments were undertaken in an attempt to find out how these anti-adhesive drugs produce their effect. Using very clean glass slides it was observed that a drop of water will spread almost indefinitely over the surface. If the glass surface had been previously exposed to a weak solution of most of the drugs listed in Table I and then thoroughly washed and dried, then the glass surface became highly hydrophobic, since a drop of water now remained as a tight drop. This strongly suggests that these substances have probably formed a monomolecular layer of orientated molecules with their hydrocarbon and hence hydrophobic moieties exposed. The glass beads used in the filter units were accordingly exposed to quinine or benadryl or cocaine and thoroughly washed and then introduced into filters. It was found that platelets in native blood passed through these filters stuck less well than to beads washed only in water. Sheets of heat-damaged amnion cells were flooded with quinine, benadryl, and cocaine and then thoroughly rinsed in saline. Blood was then trickled over the treated and untreated cells. For every hundred platelets sticking to the untreated cells only 35 to 50 platelets stuck to the treated cells. The adhesiveness was almost normal of platelets in the leading front of cocainized blood passed through the glass filter (the first drop) whereas subsequently platelets did not stick. Since the plasma and platelets were previously cocainized, this sudden decrease in platelet adhesiveness suggests that the blood-glass interface had changed. One (of many) explanations is that the cocaine rapidly coated the glass.

It thus seems likely that the anti-adhesive drugs which carry a net positive charge, stick by electrostatic forces to the negatively charged glass or damaged cell surface, and that this new surface, either by being hydrophobic or by carrying a positive charge, is not attractive to a platelet, and furthermore, this layer of drug cannot be washed off. However, when cocaine or the other drugs in plasma were spread over glass, the surface did not appear to become hydrophobic, but the surface could not be dried without the danger of denaturating any adhering protein.

Platelets when observed under the microscope

in native blood or more usually citrated plasma are mostly flat, disk shaped, and easily develop spicules. In the presence of most anti-adhesive drugs (see Table I) the platelets assumed a spherical shape. If these sphered platelets were washed in saline or native plasma they reverted to normal morphology. Furthermore, if platelets and red cells in native or citrated cocainized plasma were washed in normal native, platelet-poor plasma it was shown that the platelets became more sticky when passed through the glass filters. It was impossible to devise a situation in which the adhesiveness of cocainized platelets to a non-cocainized surface could be tested, although it is known that cocainized platelets will not aggregate. Thus, it has not been established whether a cocainized platelet can stick or not; but all these drugs, except procaine, cause platelets to sphere and this is reversible, and such washed platelets are adhesive.

The effect of these drugs on red cells was also investigated. These cells also were sphered by the anti-adhesive drugs, and at the same concentration. Higher concentrations produced haemolysis. Rouleaux formation, which is essential for fast red cell sedimentation, cannot occur when the red cells are sphered. The sphering, estimated by eye under the microscope, was more accurately reflected by the decrease in the sedimentation rate when blood with an increased sedimentation rate was mixed with the various drugs (Table I). Two washes in saline or plasma also returned them to normal. If cocaine or another of the anti-adhesive drugs was injected intradermally, a cut made over this site bleeds for 10 or more minutes. If the cut is delayed for 20 minutes after the injection then the bleeding time is normal. It can be concluded that the cocaine is rapidly removed from the area and does not stick permanently to the intact capillary endothelium. It may be concluded that these drugs can become reversibly attached to a live cell, presumably at its surface, and that the permeability of the cell membrane is altered. Skou (1954) has shown that local anaesthetics can penetrate an orientated monomolecular layer of lipids extracted from nerve cells. It is tempting to suggest that the lipophilic hydrocarbon part of these molecules can penetrate the cell surface and that this type of penetration is easily reversible, while the bonding to a damaged, more negatively charged cell or to glass is by electrostatic forces and cannot be reversed by washing.

DISCUSSION

These results gain a considerable amount of indirect support from other workers. Hellem (1960) used a very similar system to the present glass filter unit but with citrated blood. He reports that increasing the citrate concentration decreases the adhesiveness and that adhesion is not dependent upon temperature and that platelet adhesiveness is normal in many clotting defects. Furthermore, heparin injected in moderate doses, 50 mg., does not prolong the bleeding time (Hjort, Borchgrevink, Iversen, and Stormorken, 1960). Nor does it prevent the formation of white thrombi in glass arteriovenous shunts (Zucker, 1947) or in rabbits' vessels (Roskam, Hugues, and Bounameaux, 1955). Dicoumarol also has no effect on these situations. We have shown that the anti-adhesive drugs have virtually no effect on clotting. It can be concluded that the rapid platelet adhesion to 'foreign' surfaces is calcium dependent, but independent of the clotting mechanism. We found that heparin in high doses does apparently cause cell surfaces, but not glass, to become unattractive to platelets, and Hjort *et al.* (1960) have shown that it will prolong the bleeding time. This might well be due to the very strong negative charge of heparin which could bind to proteins in the damaged cell surface but not to glass. Bounameaux (1958) reports that antihistaminics inhibit viscous metamorphosis and clot retraction. Irrigating the severed small vessels in live animals with strong heparin or antihistaminics or citrate (Hugues, 1959) prevents the formation of a firm platelet plug and bleeding persists.

Hellem (1960) found that the platelets in fresh citrated plasma did not stick to glass beads but that platelets in blood did. He concludes that platelets are non-adhesive until a factor R is released from red cells by contact with a foreign surface. We agree that removal of red cells decreases platelet adhesiveness (Table III), but using native plasma some platelets still adhere (23%) and adhesion of these platelets is inhibited by Benadryl. We also found that platelet adhesion to damaged amnion cells is also decreased but not prevented by removing the red cells from native blood. It seems, therefore, that factor R is not essential for platelet adhesion, but the presence of red cells markedly increases the number of platelets that stick.

How do platelets 'stick' to damaged cells but not to intact ones, and how do the anti-adhesive drugs interfere in this process? Vroman (1958) stated that 'the impression was formed that the adhesion of platelets to a surface is merely an indicator of the absorption of plasma constituents to that surface...' The stimulating concepts of Bangham, Pethica, and Seaman (1958) are very relevant to the present problem; they suggest that one mechanism in the adhesion of cells may be due to electrostatic bridging by bi- or multivalent ions with or without the mediation proteins. It is certain that some plasma

proteins 'stick' to glass and presumably undergo some degree of reorientation (denaturation) and it is likely that they also stick to damaged cells; on the other hand, it is unlikely that any plasma proteins near an intact endothelial cell are denatured. Thus platelet adhesion may be mediated through a denatured plasma protein. There is, however, no evidence to support this concept and none to refute it except that no such protein has been identified.

The many drugs which prevent adhesion of platelets have the following characteristics in common. They carry a positive charge and have a hydrocarbon and lipophilic moiety. They are nearly all drugs of great pharmacological activity, and it is remarkable that there is considerable overlap in the activity of the heart-blocking quinidene group, the anti-malarials, the local anaesthetics, and the antihistamines. The penetration of the local anaesthetics into a nerve lipid monolayer has been mentioned, and Judah (1960) has shown that antihistamines reduce the permeability of damaged mitrochondrial (lipid-containing) membranes. The experiments reported strongly suggest that one action of these drugs is to form a layer, possibly a monolayer, on any negatively charged surface which is then less negatively charged and may be hydrophobic. (The presence of proteins may obscure this effect.) Such a surface is not attractive to a platelet, or to the intervening protein if such exists. Presumably the drug absorbed at the surface of the platelet and causing sphering will also alter its charge and then the platelet might not be attracted to a negatively charged surface. If the surface of an undamaged platelet in the blood is negatively charged, and this has not yet been demonstrated, cationic bonding with calcium could explain why it 'sticks' to a surface of a like charge and why adhesion is calcium dependent. 'Direct adhesion . . . due to the existence of a mosaic of plus and minus charges on a surface that is negative overall' (Bangham *et al.*, 1958) is another method whereby platelets could 'stick'. This concept is less likely since adhesion is calcium dependent, but the anti-adhesive drugs might also be expected to interfere with this type of adhesion.

Bangham *et al.* (1958) have tentatively identified some negatively charged groups on platelets, but they were investigating damaged platelets since these accelerated a Stypven clotting time. An undamaged platelet in native blood does not accelerate the Stypven clotting time, and it is unlikely that the platelet alters its characteristics before it sticks (Lüscher, 1959) since this is almost instantaneous (Vroman, 1958; Hugues, 1959). Bangham found the damaged platelet surface very similar to that of a lymphocyte; and the conclusion emerges that we do not know in what way an undamaged platelet surface differs from that of a lymphocyte or any other blood cell, and why a platelet and only a platelet, sticks to a negatively charged surface.

Since these drugs prevent the adhesion of platelets to a cut capillary, it may be asked whether they could also prevent the formation of a thrombus, since it has been argued (O'Brien, 1960) that the adhesion of a few platelets to a local area of damaged arterial cell is almost certainly an essential step in thrombus formation. It seems very likely that, in fact, these drugs would prevent the formation of a thrombus if they could be tolerated at an anti-adhesion concentration. However, the concentrations listed in Table I are probably all lethal. Indeed there seems to be some general physiological property attaching to these concentrations, since the same concentration prevented adhesion to glass and amnion cells and caused platelet and red cell sphering, although the intradermal injections had to be 10 times stronger. If the concentration was lowered in any of these situations then the drugs had little effect. It is clearly reasonable to hope that a drug similar to those investigated could be found that would prevent platelet adhesion and would not cause the many other effects noted, such as haemolysis, necrosis, and the well-known pharmacological effects. It seems that a more detailed knowledge of the unique surface characteristics of the intact platelet surface might enable one to find a drug that will uniquely become attached to the platelet or block the appropriate attractive sites on damaged endothelium.

CONCLUSIONS

The reported results suggest the following conclusions:

Platelet adhesiveness in the coagulation defects and in heparinized incoagulable blood is normal but adhesiveness is depressed by many drugs which have no effect on clotting. Thus, the rapid adhesion of platelets to damaged cells and to glass seems to be independent of clotting, and neither adhesion nor aggregation nor viscous metamorphosis is necessary for normal clotting.

Salicylates and procaine inhibit platelet adhesion to glass and to damaged cells—both almost instantaneous reactions—but they do not inhibit the aggregation of platelets which takes time and is probably thrombin dependent. This suggests that part, at least, of the mechanism of aggregation differs from that of adhesion.

There seems to be a complete parallel between platelet aggregation and clot retraction, both being abolished by many drugs (Table I), but both are

normal in the presence of procaine and a weak sodium salicylate solution. These findings support the concept that adhesion of platelets one to another is one of the essential prerequisites of good clot retraction. (Viscous metamorphosis is probably also essential.)

In all the various situations studied, adhesion of platelets to glass and to damaged cells has almost always run parallel, and this suggests that a roughly similar mechanism may be involved in both these forms of immediate adhesion.

Since adhesion is abolished by citrate it is likely that adhesion is calcium dependent.

Only live, fresh platelets are adhesive and no models stuck, but the unique characteristics of the platelet surface are unknown.

Glass treated with these drugs becomes hydrophobic and no longer attracts platelets. It is possible that the positively charged drugs stick electrostatically to the negative glass surface to form an orientated monolayer with the hydrocarbon part of the molecule exposed.

Most of the drugs so far studied with anti-adhesive properties delay the sedimentation rate and cause sphering of red cells and platelets. Sphering is reversible, so presumably the drug, when absorbed, alters the cell permeability and it can subsequently be washed off.

These drugs stick irreversibly to damaged amnion cells, making them unattractive to platelets, and suggesting that this type of binding may differ from that between the drug and a live cell.

It seems that any negatively charged surface, such as glass or a damaged cell, is attractive to a platelet by virtue of its negative charge, since many positively charged substances, when attached to these surfaces, prevent platelets adhering. Intact, undamaged platelets may carry a negative charge and may stick by electrostatic forces with Ca++ forming a kationic bridge. An altered plasma protein may also mediate. The same anti-adhesive drugs bind to platelets of which the charge will be altered and they may then be less 'sticky'.

This work was carried out with the aid of a grant from the Wessex Regional Board which is gratefully acknowledged. I should like to thank Dr. H. R. Ing for much pharmacological advice, Dr. J. P. Soulier, and Dr. J. Caen for allowing me to study their patients with Hageman trait in their laboratories in Paris, Dr. L. A. Hatch for assistance with the tissue culture, my volunteers, and the drug firms who supplied many drugs. My especial thanks are due to Mrs. S. B. Duke for technical assistance.

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A note on platelet adhesiveness in ischaemic heart disease

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SYNOPSIS Platelet adhesiveness was measured *in vitro* in a group of patients with ischaemic heart disease and in a group of matched healthy subjects using either 1% EDTA or 3·8% sodium citrate as anticoagulant. When sodium citrate was used platelet adhesiveness was significantly greater in the group with ischaemic heart disease. However, no difference was found when EDTA was used.

In both groups platelet adhesiveness was found to be significantly greater with sodium citrate than with EDTA.

A property of blood platelets is adhesiveness which is an important mechanism in normal haemostasis, since plugs of adherent platelets are found at the earliest stages in the formation of a white thrombus (Zucker, 1947). A tendency towards increased adhesiveness has therefore been sought in conditions characterized by pathological thrombosis. Increased platelet adhesiveness has been demonstrated by Wright (1942) postoperatively and post partum and by Moolten, Vroman, Vroman, and Goodman (1949) in a variety of thromboembolic diseases, including acute myocardial infarction. More recently McDonald and Edgill (1959) have found increased adhesiveness in angina pectoris, acute coronary insufficiency, and a recent myocardial infarction. Since the increasing incidence of ischaemic heart disease in highly industrialized white populations is said to be related to an increased predisposition towards arterial thrombosis (Morris, 1951), a further study of platelet adhesiveness in ischaemic heart disease seemed warranted.

The methods used for measuring platelet adhesiveness have not been found universally satisfactory and a variety of techniques have been described. A recent method in which adhesiveness is measured in platelet-rich plasma rather than in whole blood (Hutchison, Stark, and Chapman, 1959) has been employed in the present study.

MATERIALS AND METHODS

Two groups of male subjects who were matched for age were investigated.

Group 1 comprised 13 patients who had suffered a myocardial infarction at least four months previously and were well at the time of the investigation. They were not receiving treatment. Their mean age was 54 years.

Group 2 comprised 10 healthy subjects whose mean age was 52 years.

Blood was collected from subjects after an overnight fast with siliconed glassware into siliconed tubes kept chilled at 4°C. A two-syringe technique was used and the blood in the first syringe discarded. Duplicate samples were collected into tubes which contained either 3·8% sodium citrate or 1% EDTA, 9 ml. blood being added to 1 ml. anticoagulant. Platelet-rich plasma was obtained be spinning blood at 1,000 r.p.m. for 15 minutes at 4°C. Platelet adhesiveness was measured within 30 minutes. Aliquots, each of 3 ml., of the plasmas were rotated on a turntable at 33 r.p.m. at an angle of 45° in a refrigerator, since platelets are adhesive in citrated plasma only at low temperatures (Hellem, 1960). Samples were taken for platelet counts at a standard level in the tubes at the beginning and at half-hour intervals. The time taken for the platelet count to fall to half its initial value was taken as a measure of adhesiveness when citrate was used as anticoagulant. Since adhesiveness is greatly decreased when EDTA is used (Hellem, 1960), the percentage fall after two hours was taken as the index of adhesiveness when EDTA was used as anticoagulant. Platelets were counted directly. If more than two platelets were clumped together they were not counted.

RESULTS

The rates of fall in the number of platelets were on the whole linear over the period of observation.

Table I shows the times taken for the platelet counts to fall to half their initial values with citrate as anticoagulant and the percentage falls in platelet counts at two hours with EDTA as anticoagulant.

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TABLE I

COMPARISON OF FALLS IN PLATELET COUNTS IN PATIENTS WITH MYOCARDIAL INFARCTION AND IN CONTROLS USING 3·8% SODIUM CITRATE OR 1% EDTA AS ANTICOAGULANTS

	Citrate		*EDTA*	
	Time in Hours for Platelet Count to Fall to 50%		*Platelet Count at Two Hours (% of Initial Figure)*	
Number	*Myocardial Infarction*	*Control*	*Myocardial Infarction*	*Control*
1	1·5	3·0	96	100
2	2·0	2·5	89	88
3	3·0	5·0	96	91
4	1·0	5·0	93	100
5	3·0	3·5	74	81
6	0·5	4·0	82	93
7	2·5	2·5	90	91
8	4·0	2·0	99	75
9	1·5	2·5	80	100
10	4·5	5·0	100	90
11	4·0		95	
12	2·5		70	
13	1·0		74	
Mean ± SE	2·4 ± 0·36	3·5 ± 0·36	87·5 ± 2·86	90·9 ± 2·60
P		<0·05		>0·4

The individual figures for all subjects and the mean values for the two groups are shown. Using citrate, the mean values and standard errors were 2·4 ± 0·36 hours for the infarction group and 3·5 ± 0·36 hours for the control group. This difference was statistically significant ($P = <0·05$). Using EDTA, the mean percentage fall at two hours was to 87·5 ± 2·86% of the initial figure for the infarction group and to 90·9 ± 2·60% for the control group. This difference was not significant.

To determine the effect *in vitro* of the two anticoagulants on platelet adhesiveness in general, the two-hour platelet counts in all 23 subjects were calculated as a percentage fall. This was done for citrate and EDTA. The mean fall was to 63% of the initial figure with citrate and to 89% with EDTA, and this difference was statistically significant ($P = <0·001$). Platelet adhesiveness was therefore greater when citrate was used.

At the completion of each experiment a sample of plasma was obtained with a Pasteur pipette from the bottom of the tube and examined microscopically. Whenever citrate was used many clumps consisting of large numbers of platelets were seen. With EDTA very few clumps consisting only of a few platelets were found.

DISCUSSION

When citrate was used as an anticoagulant for the collection of blood, the platelets of a group of patients with a myocardial infarction were found to adhere significantly more than the platelets of healthy subjects matched for age. This is in agreement with the findings of McDonald and Edgill (1959) who investigated a group of patients with a more recent infarction than in the present group. McDonald and Edgill did state, however, that even at a later stage the platelets of such patients remained abnormally adhesive. However, when EDTA was used as anticoagulant in the present experiments no such difference was found.

The interpretation of these results is difficult since the mechanism of platelet adhesiveness has not been entirely established. Hellem (1960) has demonstrated the influence of red blood cells on adhesiveness. He showed that a substance, named factor R, was released from red cells, and that in the presence of factor R the non-adhesive platelet in citrated platelet-rich plasma became adhesive. This, however, does not explain the findings that platelets which are not adhesive in citrated plasma at room temperature become adhesive at low temperatures and that platelets suspended in saline are adhesive even at room temperature (Hellem, 1960). It is possible that the increased adhesiveness of platelets at low temperatures is related to the morphological alterations which have been found to occur when platelets are stored at 4°C. (Zucker and Borrelli, 1954).

The mechanisms responsible for the difference found between the two groups with citrated platelet-rich plasmas is therefore obscure. In addition aggregation of platelets contributed to the falls in platelet counts. However, Hellem (1960) considers that the mechanisms responsible for aggregation and adhesiveness are related, since he found that factor R produced platelet adhesiveness in low concentration and aggregation in high concentration. The finding of similar platelet adhesiveness in the two groups when EDTA was used as anticoagulant is probably of little significance, since very little adhesiveness could be demonstrated with this anticoagulant. This is in agreement with Hellem (1960) and may also be related to an alteration in platelet morphology (Zucker and Borrelli, 1954).

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The application of *x*-ray diffraction and electron microscopy to the study of human peripheral nerve obtained by biopsy

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SYNOPSIS Human peripheral nerves obtained by biopsy from patients suffering from neuromuscular disorders have been studied by *x*-ray diffraction and electron microscopy. Few abnormal diffraction patterns have yet been recorded and their significance is not yet established. Electron micrographs have revealed wide variations in the numbers of myelinated fibres included in the nerve trunks and have facilitated a detailed study of the Schwann cell-axon relationships in the large numbers of unmyelinated fibres always present. Some indications of demyelination have been encountered.

In the past, the range of abnormalities detectable in biopsy specimens of cutaneous nerves has been severely limited. Morphological changes detectable by the light microscope have been largely restricted to degenerative swelling of myelin sheaths and axons, loss of myelin as observed in 'myelin preparations', and loss of non-myelinated fibres as seen in silver preparations. Little information relating to the Schwann cells could be obtained and, as will be shown, the smallest axons could not be seen with the light microscope. Furthermore, little structural detail could be discerned in individual nerve fibres. This applies particularly to axons, which stain almost uniformly in silver preparations.

This paper reports a preliminary attempt to extend the scope of biopsy studies of peripheral nerve by introducing *x*-ray diffraction and electron microscope techniques.

MATERIALS AND METHODS

The specimen studied was almost invariably a small branch of the medial antebrachial cutaneous nerve of the forearm taken in the course of muscle biopsy in a variety of neuromuscular diseases. In only a few cases was there any evidence of disturbed cutaneous sensation.

A 2 cm. length of nerve was isolated with great care and cut into four pieces which were immediately immersed in chilled saline or fixative. The specimen for diffraction studies was immersed in 0·9% saline, those for electron microscopy in 2% OsO_4 in veronal-acetate buffer, *p*H 7, or 1% $KMnO_4$ in veronal-acetate buffer, *p*H 7, that for paraffin embedding for light microscopy in the buffered osmic acid, and that for haematoxylin and eosin staining for light microscopy in 10% formol saline.

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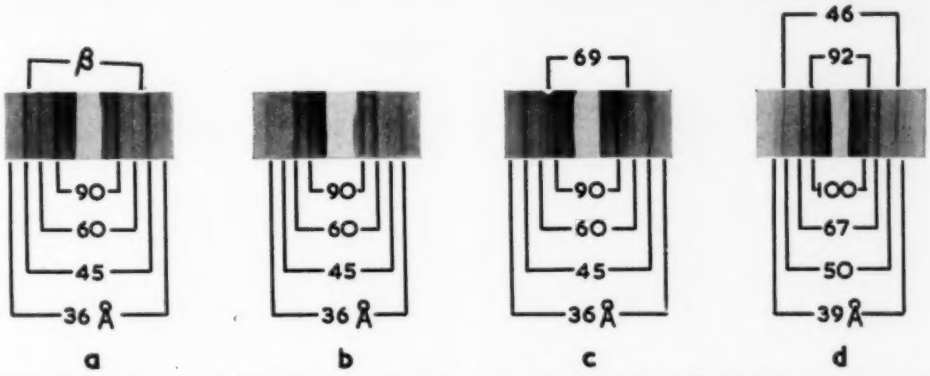
So far, specimens of nerve from 75 patients have been made available for *x*-ray diffraction and electron microscope studies.

The nerve selected for diffraction studies was stored at about 4°C. until the diffraction pattern could be recorded. The delay in obtaining the diffraction pattern was never more than six hours and frequently less than one hour. As a check on the possibility of degenerative changes taking place during the period of delay before recording the diffraction patterns, rat sciatic nerves were kept under similar conditions for comparable periods, and the diffraction patterns subsequently obtained were compared with those from freshly isolated preparations. In addition, specimens of human nerve which had been examined very soon after isolation were frequently stored and re-examined after several hours to see if such storage resulted in a change in diffraction pattern.

For the purpose of recording the diffraction patterns the nerve samples were enclosed in close-fitting, thin-walled, glass capillary tubes filled with physiological saline. A low-angle *x*-ray diffraction camera of a type previously described (Finean, 1956) was used, and a standard exposure of one hour at a specimen to film distance of about 150 mm., using a slit collimating system, was adopted.

Specimens for electron microscopy were fixed for two hours in OsO_4 or three hours in $KMnO_4$, washed in saline, and left in 70% ethyl alcohol at about 4°C. overnight. The dehydration was completed in 90% and



Electron micrographs were selected from studies relating to five Werdig cases, namely, Case 1, dermatomyositis, Case 2, Werdig-Hoffmann disease, Case 3, an obscure, slowly progressive peripheral neuropathy, Case 4, sensory neuropathy, and Case 5, dystrophia myotonica.

FIG. 1. *Low angle x-ray diffraction patterns obtained from human peripheral nerve. a, Normal pattern; b, c, d, abnormal patterns. The numbers refer to the diffraction spacings calculated for each band in the pattern assuming that they arise from the CuKα radiation (λ = 1·54 Å). They are grouped so as to distinguish bands related to the main fundamental spacings,* i.e., *those below the pattern. As unfiltered Cu radiation was used in these experiments diffraction bands related to CuKβ radiations (λ = 1·39 Å) can occasionally be distinguished. Such a diffraction is indicated as β in a, and corresponds to the 45 Å diffraction.*

absolute alcohols, and the specimens were embedded in Araldite casting resin. Thin sections were cut with the aid of Porter-Blum or Philips ultramicrotomes and examined in a Siemens electron microscope, type Elmiskop I.

RESULTS

Little reference will be made to the conditions from which the patients were suffering, as in this preliminary report the object is simply to show the scope of the methods and their potential value rather than to attempt to identify pathological changes. A more detailed treatment of individual cases will be included in case reports which will be published separately.

X-RAY DIFFRACTION Of the 75 nerve specimens examined by low-angle diffraction methods, 45 gave a pattern which, from a comparison with those of other mammalian peripheral nerve (Finean, 1960) such as rat sciatic nerve, could be regarded as normal. This pattern showed four reflections which could be accounted for as the second, third, fourth, and fifth order diffractions of a fundamental period of 180 Å. The first order diffraction was not readily detectable under the conditions used in these experiments. An example of such a diffraction pattern is reproduced in Fig. 1a.

Of the remaining nerve specimens, 17 yielded no detectable diffraction and 13 gave abnormal patterns. The most common abnormality was an increased intensity of the third order diffraction accompanied by a decrease of the fourth order (Fig. 1b), but a few preparations gave diffraction spacings (Fig. 1c and 1d) not present in the normal pattern. No straightforward relationship between these abnormal patterns and the clinical condition of the patients from whom the nerves were taken is yet apparent.

ELECTRON MICROSCOPY So far about 50 of the preparations have yielded useful electron microscope data but only 10 have been studied in detail.

All preparations which had given intense diffraction patterns yielded electron micrographs showing large numbers of well-myelinated nerve fibres, always accompanied by a moderate proportion of unmyelinated ones.

In general, there was a close correspondence between the intensity of the diffraction pattern of the fresh tissue and the abundance of myelinated fibres as seen in electron micrographs of nerve cross-sections, although in a few cases myelinated fibres were detected in electron microscope preparations when the equivalent *x*-ray diffraction specimen had yielded no pattern.

The myelin layers, as seen in both OsO_4-fixed and in $KMnO_4$-fixed preparations, showed exactly the same features as have already been described for other mammalian peripheral nerves (Fernández-

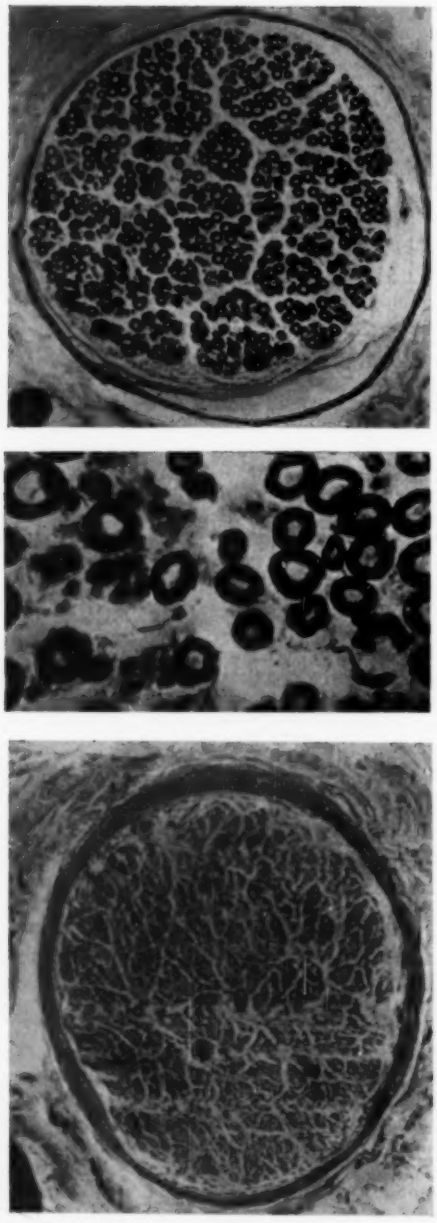


FIG. 2. *Photomicrographs obtained from cross sections of OsO_4-fixed and paraffin-embedded preparations of biopsy material. a Case 1 × 200, b Case 1 × 900, c Case 4 × 400*

Morán, 1957; Fernández-Morán and Finean, 1957; Finean and Robertson, 1958; Robertson, 1959, 1960; Sjöstrand, 1960). The continuity between the Schwann cell membrane and the myelin layers (the mesaxon) was frequently observed, and it was clear that the process of myelination had been essentially the same as has been established for other species (Geren, 1954; Robertson, 1959; Peters and Muir, 1959). The relationships between nerve axons and Schwann cells in the unmyelinated fibres were also similar to those previously reported (Causey, 1960; Gasser, 1958; Robertson, 1959).

The variation in the proportion of myelinated to unmyelinated fibres provided the most striking feature of these electron microscope studies. In several preparations very few myelinated fibres were to be seen, and in one case none at all. Unmyelinated fibres, however, still appeared numerous, and there was an apparently normal relationship between axons and Schwann cells. Many of these poorly myelinated nerve specimens will be considered in greater detail in other publications but some electron micrographs relating to a few cases are reproduced here in order to illustrate the general points made.

Fig. 3 shows low-magnification electron micrographs selected to illustrate the variations in the composition of nerve bundles encountered in these studies. Fig. 3a is taken from an adult nerve containing numerous well-myelinated fibres. Photomicrographs from the same tissue are shown in Fig. 2a and b.

In this preparation the myelinated nerve fibres accounted for at least 50% of the tissue volume but unmyelinated fibres were freely interspersed, frequently concentrated into large bundles.

In specimens taken from infants under 1 year old myelinated fibres were far less abundant (Fig. 3b), and both axons and myelin sheaths were much smaller than in the adult tissue. Unmyelinated fibres appeared to be far more numerous but the effect was certainly enhanced by the fact that myelinated fibres were small and accounted for only a small proportion of the tissue volume.

Fig. 3c shows only a few myelinated fibres but this micrograph was obtained from a specimen taken from an adult suffering from an obscure, slowly progressive, peripheral neuropathy. A striking feature of the micrographs obtained from this preparation is the prominence of numerous large axons surrounded by regions of unusually low density. The distribution of such regions is reminiscent of the distribution of well-myelinated fibres in normal adult tissue, and these low density regions may well reflect the degeneration of large fibres (see also Bunge, Bunge, and Ris, 1960). Fig. 6b is a

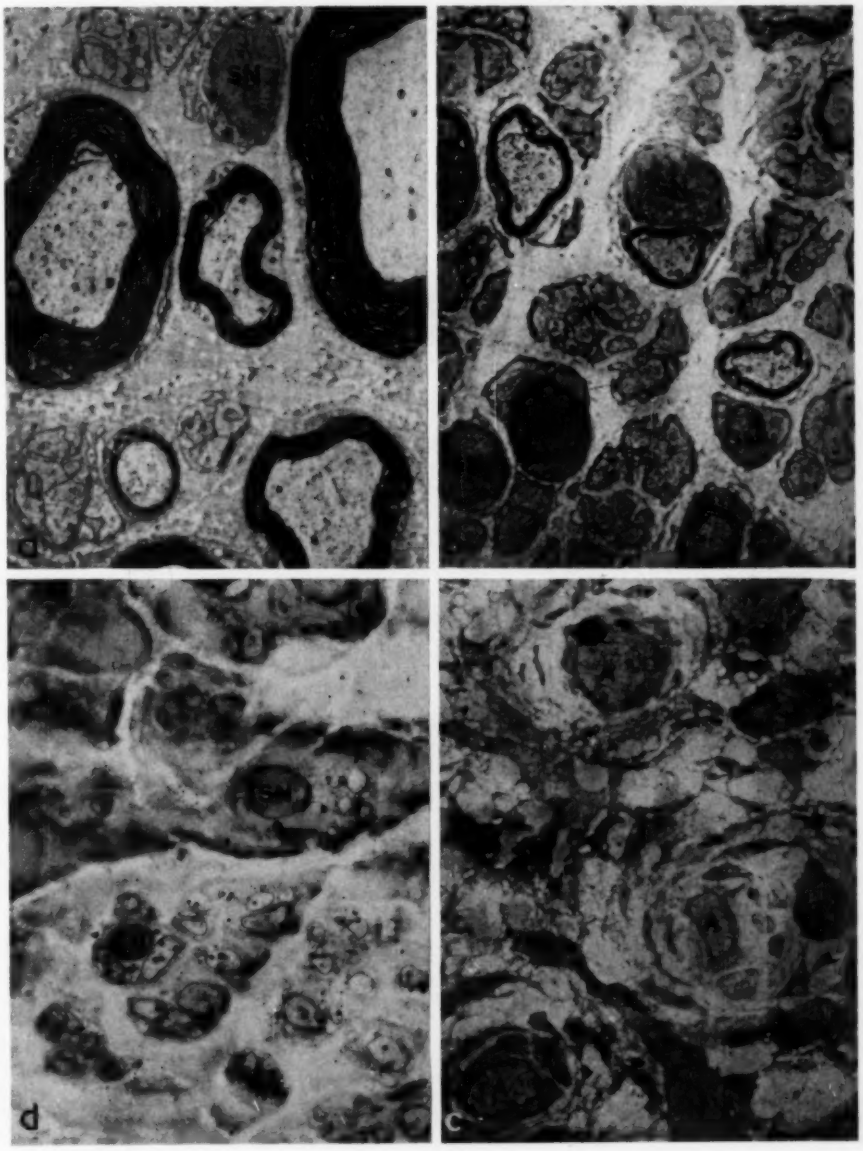


FIG. 3. *Low magnification (× 5,000) electron micrographs of cross sections obtained from biopsy specimens. A number of Schwann cell nuclei (SN), unidentified nuclei (N), and axons (A) are labelled. a Case 1 OsO_4-fixed b Case 2 OsO_4-fixed c Case 3 $KMnO_4$-fixed d Case 4 OsO_4-fixed*

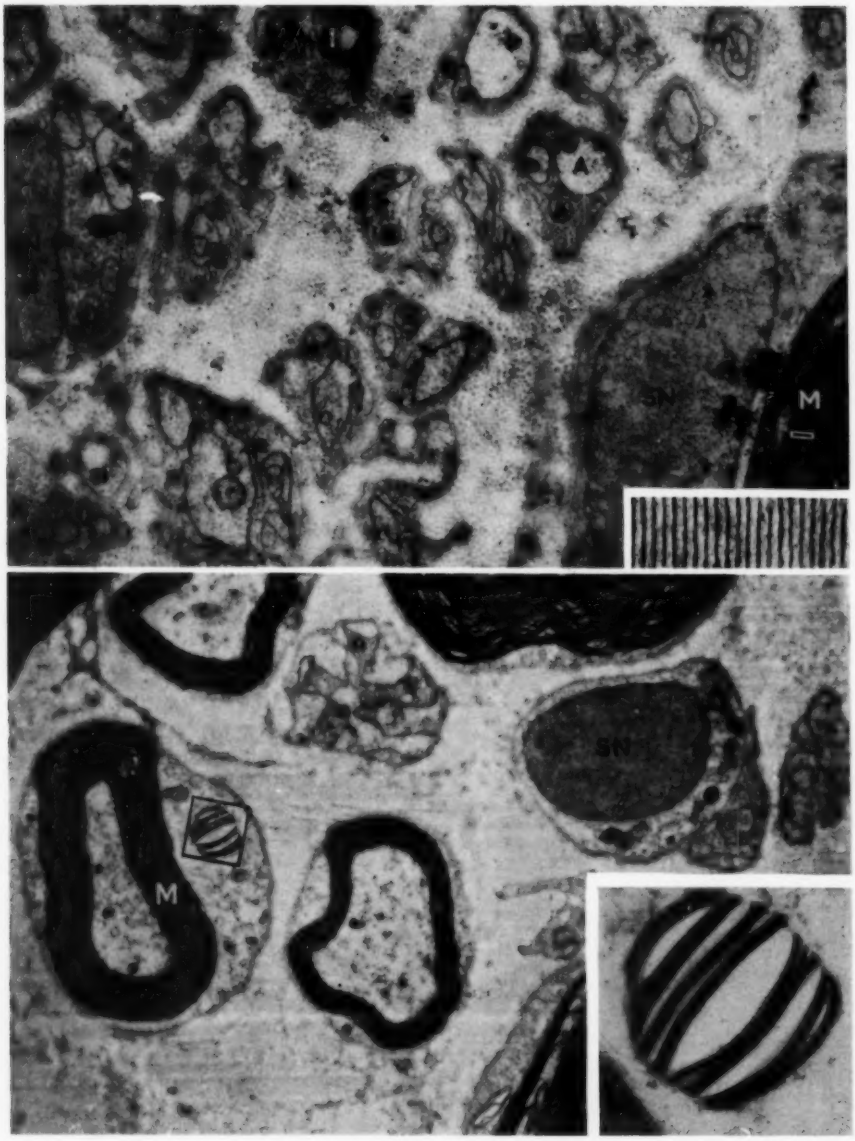


FIG. 4. *Electron micrograph (× 15,000) from Case 5 showing a portion of a large myelinated fibre (M) (inset myelin layers × 160,000) and a group of unmyelinated fibres with examples of large and small axons (A).*

FIG. 5. *Electron micrograph (× 10,000) from Case 1 showing a typical 'normal' distribution of myelinated and unmyelinated fibres and including an unusual layered body (inset × 40,000).*

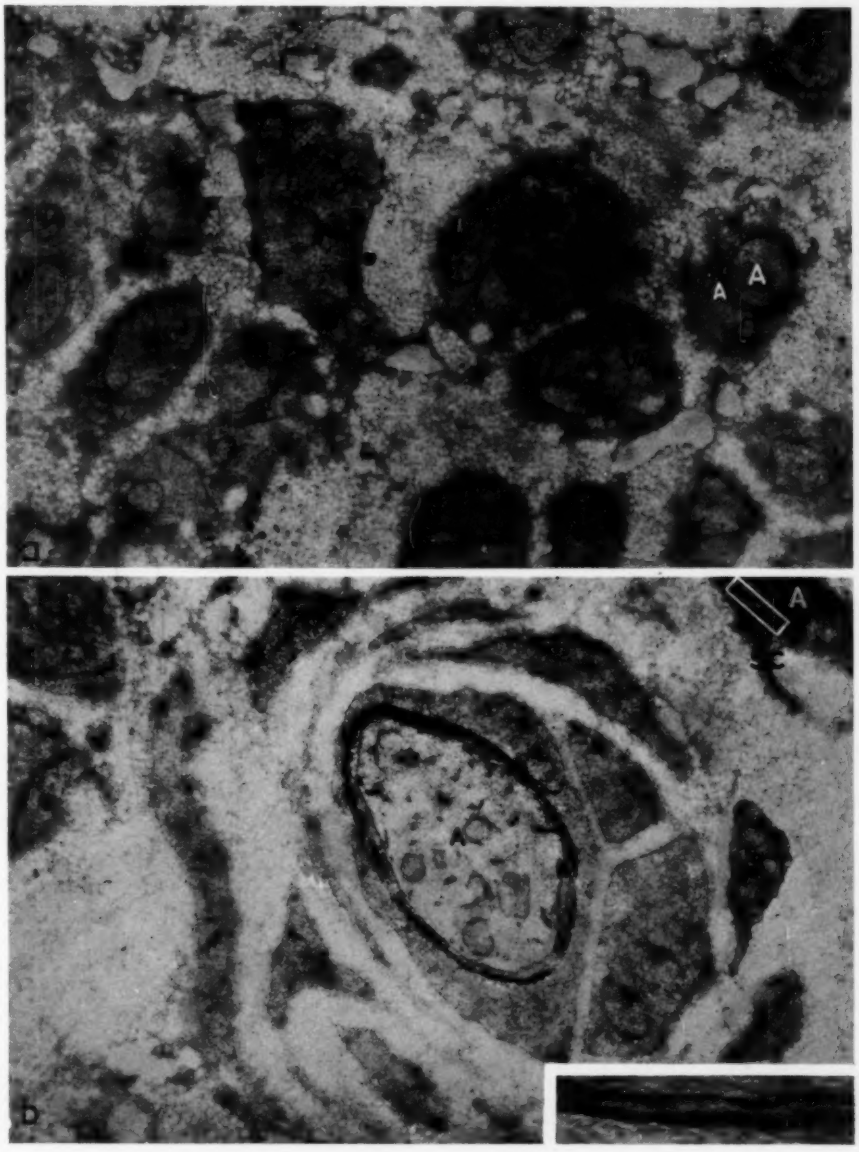


FIG. 6. *Electron micrographs from Case 3. a A group of unmyelinated fibres* × *15,000. b A sparsely myelinated fibre located in a region of low density.* × *40,000. Inset is a high magnification* (× *160,000*) *electron micrograph showing detailed structure of apposed axon and Schwann cell membranes.*

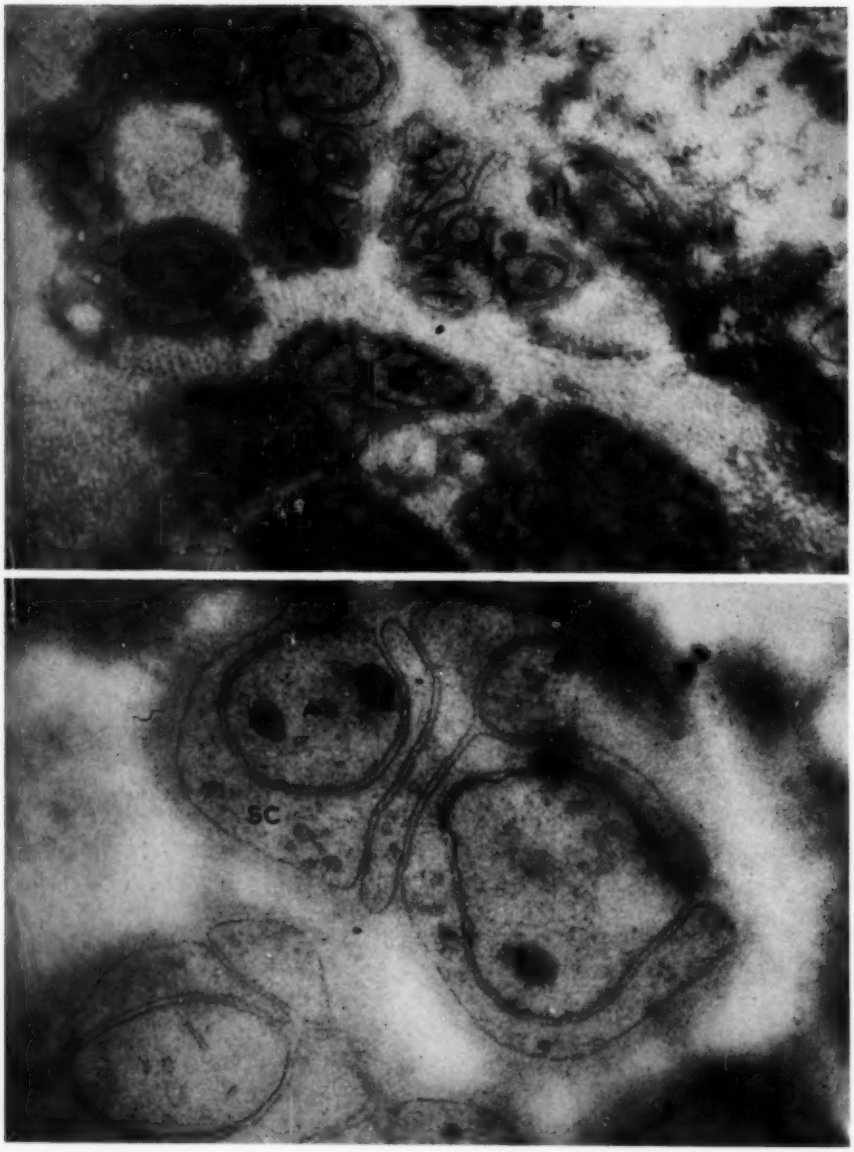


FIG. 7. *Electron micrographs from Case 4. a Unmyelinated fibres* × *20,000. b Unmyelinated fibres* × *60,000. In one nerve fibre Schwann cell (S.C.), axon (A), and mitochondria (Mi) are distinguished.*

relatively high-magnification electron micrograph which shows one of the few myelinated fibres detected in this case. Very few myelin layers surround the axon, and the general appearance is that of a nerve in an early stage of myelination. However, the region around the nerve fibre shows an unusual array of profiles, presumably of cell processes, and also a low density such as has already been considered as possibly arising from a demyelination of some of the larger axons. This fibre may, in fact, be in the process of remyelination. Unmyelinated fibres are also present, and their distribution is similar to that of the normal adult tissue. A group of such fibres is shown in Fig. 6a.

A case of sensory neuropathy in which light microscopy, electron microscopy, and *x*-ray diffraction all showed a complete absence of myelinated fibres is illustrated in Figs. 2c, 3d, and 7. Unmyelinated fibres are numerous, but they are more widely distributed than in the case illustrated in Fig. 3b. There are no obvious signs of degenerative changes, but again this is an adult nerve and the clinical history suggests that an extremely slow degeneration has probably taken place. The higher magnification electron micrographs (Fig. 7) show a variety of apparently normal axon-Schwann cell relationships among the unmyelinated fibres and include some examples of a single axon embedded in a Schwann cell, a situation that might be expected to lead to myelination in normal circumstances.

The higher magnification electron micrographs also serve to illustrate some of the more detailed structural features revealed in biopsy preparations by the application of electron microscopy.

Detail of the myelin layering is readily observed (Fig. 4 inset) but so far no abnormalities of layering have been detected in these biopsy specimens. Electron microscope preparations corresponding to those fresh nerve specimens which had yielded abnormal diffraction patterns showed no comparable abnormalities in the appearance of the myelin layers. However, one unusual feature which has been observed in several of the human specimens is a layered body, usually of circular or elliptical outline, which is located in the Schwann cell cytoplasm adjacent to the myelin sheath (Fig. 5). The significance of this body is not yet understood.

Electron microscopy has also revealed the presence of minute axons included in the bundles of unmyelinated fibres (Figs. 4, 6a, and 7a). It is possible to examine in detail the relationships between the surfaces of the axons and the Schwann cell, and the inset in Fig. 6b shows the detail of closely apposed axon and Schwann cell surfaces as revealed in $KMnO_4$-fixed preparations. The interaction of Schwann cell and axon is the central theme of the myelination process and is the main object of attention in the present studies of abnormal nerve specimens.

DISCUSSION

X-RAY DIFFRACTION The low-angle *x*-ray diffraction technique would appear to provide a rapid method of assessing the extent of myelination in a nerve trunk. In the present studies, the exposure given was one hour and the starting of the exposure was often delayed for technical reasons but it is possible to obtain an adequate diffraction pattern in about 10 minutes, and there is no reason why this exposure should not be made immediately after the nerve has been isolated.

The significance of the abnormal diffraction patterns is still uncertain. An intensification of the third order diffraction such as some of the human preparations revealed can be produced experimentally by changing the tonicity of the immersion medium and even by leaving nerve preparations in physiological (0·9%) saline for long periods. However, it should not occur in normal preparations within six hours if stored under favourable conditions. The results so far would therefore suggest that in some cases the myelin is either already modified before isolation or is in a condition such that deterioration occurs rapidly after isolation, even if stored under favourable conditions. The absence of corresponding abnormalities in the myelin layers as seen in the electron micrographs might mean that the changes have taken place after isolation. However, no definite conclusions will be drawn until further examples have been rigorously investigated. A similar situation also exists in relation to the few preparations which have yielded diffraction spacings not present in the 'normal' pattern.

The first conclusion to be drawn from the electron microscope studies is that the structure and method of formation of the myelin sheath in human peripheral nerve is essentially similar to that already well established for other species. The axon-Schwann cell relationships observed in the case of the unmyelinated fibres is also of the type previously described for peripheral nerve from many species.

From the point of view of the study of human nerve fibres, both for the functional correlations and for the recognition of pathological changes, the most striking features are the complex pattern of distribution of myelinated and unmyelinated fibres and the amazing variety of detail presented by the Schwann cell-axon interactions among the unmyelinated fibres. The myelinated fibres in normal adult tissue show a fairly sharp distinction between large, well-myelinated fibres and groups of smaller

fibres with relatively thin myelin sheaths. Among the unmyelinated fibres there is a remarkable variation in the size of the axons and the numbers enfolded by each Schwann cell. These morphological variations may be related to different functions, but we do not yet know how to recognize by these structural features or by any other means the fibres transmitting pain, thermal and touch sensations, or those forming part of the autonomic nervous system. Such correlations can hardly be obtained through studies in animals, where the assessment of sensory function is almost impossible and the delicate interruption by disease of individual modalities of sensation virtually unrecognizable. Such information may eventually be obtained through correlations of electron microscope data obtained from human biopsy material with analyses of the reactions of the patients to various stimuli, but a great deal of systematic work will be required before any significant conclusions can be reached. It is hoped that our findings will be considered encouraging evidence of the potential value of electron microscopy in the pursuit of such correlations.

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A haemagglutination test for anti-Panton-Valentine leucocidin in serum: an interim report on 200 cases

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SYNOPSIS The test is an application of the method originally described by Boyden (1951) and adapted by Stavitsky and Arquilla (1958). Sera which contain anti-Panton-Valentine leucocidin agglutinate the prepared red cells, often to a very high dilution, and this has provided a useful serological test for the diagnosis of staphylococcal disease in orthopaedic lesions. The test is quicker as a routine investigation than the bioscopic method previously described by Towers and Gladstone (1958).

MATERIALS AND METHOD

SHEEP CELLS Defibrinated blood or cells taken into Alsever's solution are suitable. The cells are thrice washed in saline and used only if the third washing shows no haemolysis. Packed cells, 0·5 ml., are made up to about 20 ml. in buffered saline *p*H 7·2 and adjusted so that 1 ml. suspension + 9 ml. ammoniated water give a reading of 30 on an Eel photoelectric colorimeter. These standardized cells are ready for tannic acid treatment but will keep for 24 hours at 4°C.

BUFFERED SALINE Buffered salines at *p*H 6·4 and 7·2 are made by mixing 100 ml. sterile saline with 100 ml. of the appropriate 0·15 M buffer.

N/100 NORMAL RABBIT SERUM Normal rabbit serum, 1 ml. in 100 ml. sterile saline.

TANNIC ACID Stock 1% Merck reagent grade. Store at 4°C. Dilute 1:20,000 in saline for use.

LEUCOCIDIN The crude concentrate of Panton-Valentine leucocidin was kindly supplied by Gladstone and Woodin at Oxford. It was used in the concentration of 50L. + units/ml. in the proportion of 0·02 ml. to 16 ml. tanned erythrocytes.

SERA These were inactivated at 56°C. for 20 minutes before use.

PREPARATION OF TANNED CELLS Standardized sheep cells, 16 ml., and 16 ml. 1:20,000 tannic acid are mixed and left at 4°C. for 30 minutes, then centrifuged gently, washed with 16 ml. *p*H 7·2 buffered saline, and resuspended in 16 ml. saline. It is important to spin gently at all stages of preparation of the erythrocytes to ensure a final even resuspension of sensitized cells.

SENSITIZATION OF TANNED CELLS. Buffered saline, 64 ml. at *p*H 6·4, and 0·02 ml. Panton-Valentine leucocidin crude concentrate and 16 ml. tanned red cells are mixed in that order and kept on the bench for 15 minutes. The cells are centrifuged gently, washed once with 32 ml. N/100 normal rabbit serum and resuspended in 16 ml. N/100 normal rabbit serum ready for use.

HAEMAGGLUTINATION TEST Test tubes of 3 × ½ in. are used, and 0·5 ml. quantities in a dilution of 1 in 1,000 to 1 in 256,000 using N/100 normal rabbit serum as the diluent for the inactivated sera are set up. A known serum control is included in each batch and two tubes containing 0·5 ml. diluent only.

Sensitized cells, 0·06 ml., are added to each of the nine tubes in each test row, and to the two control tubes of diluent. The rack is well shaken and left on the bench overnight. The tubes are read over a concave mirror and graded according to the pattern formed by the agglutinated cells, in which O = smooth round button of cells; + = crenated button or a heavier ring round each button with a smooth interior; ++ = definite agglutination, or with a heavier peripheral ring than in +; +++ = discrete granular agglutination or a folded edge to mat. The titre is the last tube to show a positive result. Perspex racks or flat-bottomed tubes are unsuitable, as no consistent pattern is obtained.

RESULTS

The results on the 25 sera estimated by both the

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bioscopic and haemagglutination methods are shown in Table I.

TABLE I

COMPARISON OF BIOSCOPIC AND HAEMAGGLUTINATION METHODS

Bioscopic Method	*Haemagglutination Method*
Normal range up to 10 12 sera	Titre of 8 or less 10 sera (+ 2 sera giving titre 16)
Raised values over 10 13 sera	Titre of 16 or over 12 sera (+1 serum with titre of 8)

The haemagglutination test results on the 200 sera are given in Table II, and are shown in relation to the anti-α haemolysin test, which is the most widely accepted serological test for staphylococcal disease.

TABLE II

RESULTS ON 200 SERA ESTIMATES FOR ANTI-PANTON-VALENTINE LEUCOCIDIN COMPARED WITH ANTI-α HAEMOLYSIN

Anti-Panton-Valentine Leucocidin (serum titre)	*Anti- α Haemolysin (normal value)*	*Anti- α Haemolysin (values above 2 units)*
Group I		
1 or less	30 sera	1 serum
	14 miscellaneous	osteomyelitis
	10 normal controls	
	4 bone tuberculosis	
	2 bone tumours	
Group II		
8 or less	67 sera	11 sera
	31 miscellaneous	4 tuberculosis with secondary staphylococcol infection
	10 normal controls	
	10 tuberculosis	7 chronic osteomyelitis
	4 malignant disease	
	12 chronic osteomyelitis	
Group III		
16 or over	16 sera	39 sera
	5 osteomyelitis	1 normal control (recent boils)
	2 malignant disease	
	9 still under investigation; 6 probably osteomyelitis	38 osteomyelitis

It will be seen that of the total 200 sera examined, 69 were from cases of osteomyelitis; of these 69 cases, 49 (71%) gave an end-point at a dilution of 1 in 16,000 or over. The 19 chronic cases in Group II were all of long standing admitted for orthopaedic surgery.

Acute osteomyelitis, or a flare-up of old disease, seems to be reflected in the higher titres of anti-Panton-Valentine leucocidin, the exception being so far serum obtained from a child from whom a staphylococcus was recovered subsequently from the spine. The anti-α haemolysin in this case rose quickly to 16 to 20 units/ml. without any detectable anti-Panton-Valentine leucocidin over three months, and the *Staph. pyogenes* itself has since been shown to produce no Panton-Valentine leucocidin.

The usefulness of an additional test for staphylococcal disease in cases where material has not been available for bacteriology has already been stressed (Towers and Gladstone, 1958). Figs. 1 and 2 show

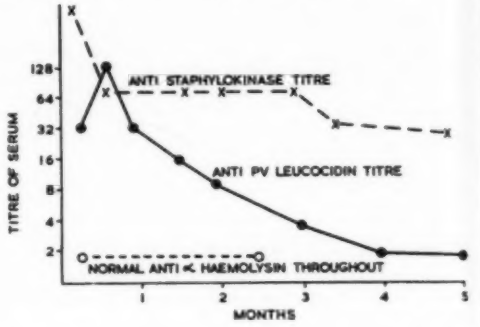

FIG. 1. *A man aged 40, with destructive lesion at D 10. No material was available for culture.*

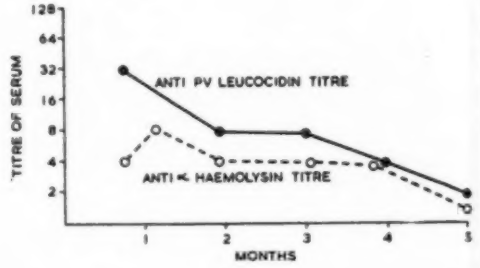

FIG. 2. *Case 2. A woman, aged 33, for 10 months suffered backache and for one month acute pain. No material was available for culture.*

how the haemagglutination estimation has helped in the diagnosis of two cases of spinal osteomyelitis of unknown origin.

In both cases there was a history of back pain for several months before admission as tuberculous spine. In one the anti-α haemolysin remained at normal levels throughout the five-month stay in hospital, in the other there was a rise of 4 units/ml. with a gradual return to normal. In both the anti-Panton-Valentine leucocidin titres were raised on admission and showed a gradual return to normal.

The obvious criticism of the method presented here is that a crude extract of Panton-Valentine leucocidin has been used as a sensitizing agent, and

the results given as the end-point of the dilution of serum, but this is an interim report only.

Woodin (1959) has now shown that anti-Panton-Valentine leucocidin is separable into two fractions, the fast and slow-moving components, and gives the method for their preparation and separation. Tests so far made with these purified components have shown that the results obtained with slow-component sensitized erythrocytes gives titres similar but lower than those obtained using the crude extract.

It is hoped to follow this series with another, in which the purified slow component is used as the sensitizing antigen. In view of the increasing widespread interest in staphylococcal disease it would be highly desirable to have a standard preparation of Panton-Valentine leucocidin generally available. This would enable comparative data on anti-Panton-Valentine leucocidin to be collected from various forms of staphylococcal disease.

It is a pleasure to thank Dr. G. P. Gladstone and Dr. A. M. Woodin for gifts of crude concentrates of Panton-Valentine leucocidin and fast and slow components of the more purified extract; Dr. H. Schwabacher of Watford who very kindly undertook the independent estimation of the 25 sera by the bioscopic method: and finally to Dr. C. H. Lack for continued interest and help in the preparation of this report.

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Quantitative determination of haemoglobin A_2 using paper electrophoresis

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SYNOPSIS A quantitative paper electrophoresis method for the separation of haemoglobins A and A_2 is described. The possible sources of error and the accuracy of the method have been investigated. The normal range for haemoglobin A_2 by this method is 1·3% to 4·3% of the total haemoglobin concentration. Good clinical correlation has so far been obtained.

With the migration of peoples, some of whom may be carriers of the thalassaemia gene, and the rising standards of medicine which today require the intensive laboratory study of anaemias of unknown aetiology, the necessity for further laboratory assistance in the diagnosis of thalassaemia has become obvious. A considerable amount of work has been carried out since Kunkel and Wallenius (1955), using starch block electrophoresis, first isolated haemoglobin A_2, and showed (Kunkel, Ceppellini, Müller-Eberhard, and Wolf, 1957) that raised values are usually found in thalassaemia minor. Gerald and Diamond (1958) suggested that a raised A_2 level combined with microcytosis are essential criteria before a diagnosis of thalassaemia minor can be established. The disadvantages of the starch block method are that it is relatively time consuming, and the number of samples that can be estimated is correspondingly restricted. Cradock-Watson, Fenton, and Lehmann (1959) demonstrated that good separation of A and A_2 haemoglobin components can be obtained on paper using a buffer containing Tris (trishydroxymethyl aminomethane) and E.D.T.A. acid. The purpose of this paper is to show the results of the combination of the Tris buffer method for the separation of the haemoglobins with the quantitative method for the estimation of proteins using bromphenol blue dye (Wilkinson and Wilkinson, 1960), to describe the modification of techniques, and to assess the reliability of the results.

TECHNICAL METHODS

All blood specimens were collected using di-potassium sequestrene as an anticoagulant. Haemolysates were prepared by the distilled water and toluene method of Singer, Chernoff, and Singer (1951). The foetal haemoglobin levels were estimated when required, and the haemolysates were then converted to carbonmonoxy haemoglobin.

Paper electrophoresis was carried out in vertical tanks (Flynn and de Mayo, 1951), using nine paper strips (Whatmann No. 3 MM) per tank, each measuring 25·5 × 4·0 cm. The shorter paper length of 25·5 cm. compared with the 36 cm. of Cradock-Watson *et al.* (1959) gave a higher voltage gradient with better separation under our conditions. Power was supplied from a 255 constant voltage power pack, the current being 2 milliamperes per strip applied for 16 hours. The Tris buffer as described by Cradock-Watson *et al.* (1959) was used and adjusted to *p*H 8·6 with boric acid. On each paper 20 c. mm. of haemolysate was applied with a micropipette, and the paper immediately moistened with buffer on both sides to within a quarter of an inch of the point of application. This method of application was found to give the sharpest definition of the bands. After completion of the run, the papers were dried in an oven at 90-100°C. and fixed for 10 minutes in an ethanol solution containing 10% mercuric chloride and 10% glacial acetic acid. It was found to be essential to discard the fixative after each use, as prolonged standing with the buffer salts gave rise to some precipitation, which resulted in artificially high values for the smaller components. Accordingly, only small quantities sufficient to moisten the papers were used on each occasion.

After fixation, the papers were dried, washed in two changes of distilled water for 10 minutes, and then thoroughly dried. Staining was then carried out for 10 minutes with an ethanol solution containing 1% bromphenol blue and 1% glacial acetic acid. A solution of 0·5% acetic acid in 25% ethanol was used for washing, as the addition of ethanol to the washing solution was found to give more rapid elution of the excess dye. Three washes of 10 minutes and two of 15 minutes were found to give a stable green colour. Wilkinson and Wilkinson have shown the importance of reaching this stable

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point. After drying, the papers were marked and divided at the point of least colour between the two bands. The dye from the A_2 band was eluted with 10 ml. of 1·5% (w/v) sodium carbonate in 50% (v/v) methanol, and that from the main A band with 30 ml. of the same solution. This latter solution was subsequently diluted 11 times to bring the optical density well within the most sensitive region of the instrument. The optical densities of the eluates were measured at 595 mμ in a Unicam S.P. 400 spectrophotometer.

RESULTS

ELECTROPHORETIC SEPARATION OF HAEMOGLOBIN A_2 Fig. 1 shows typical electrophoretic patterns obtained from both normal and thalassaemia minor haemolysates.

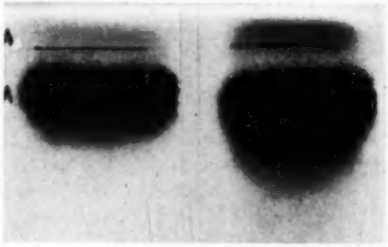

FIG. 1. *Typical electrophoretic patterns obtained from the blood of normal and thalassaemia minor patients.*

LINEARITY OF DYE UPTAKE By serial dilutions of the dye it was demonstrated that Beer's law was obeyed over the range of densities in use, and that the density readings were well within the maximal sensitivity range of the instrument. Wilkinson and Wilkinson have demonstrated that this linearity may not always hold, particularly at higher density values.

Standard curves for haemoglobins A and A_2 were constructed by subjecting 20 c.mm. samples of varying concentrations of haemoglobin solutions to electrophoresis. The concentration of the solutions under test ranged from 1 to 19 g. of haemoglobin per 100 ml. of haemolysate. Blanks were also tested and the values deducted from the readings of the other components. These results are shown in Figs. 2 and 3, and it can be seen that departure from linearity of dye uptake by the haemoglobin A occurs at concentrations above 7 g. of haemoglobin per 100 ml. of haemolysate. As the A_2 band usually comprises less than 10% of the haemoglobin A band, linearity would be anticipated throughout the range of solutions applied, and that above 7 g. of haemoglobin per 100 ml. of haemolysate, a relative increase in the percentage haemoglobin A_2 would occur. After this experiment all haemolysates applied have ranged from 4·5 g. to 7 g. of haemoglobin per 100 ml.

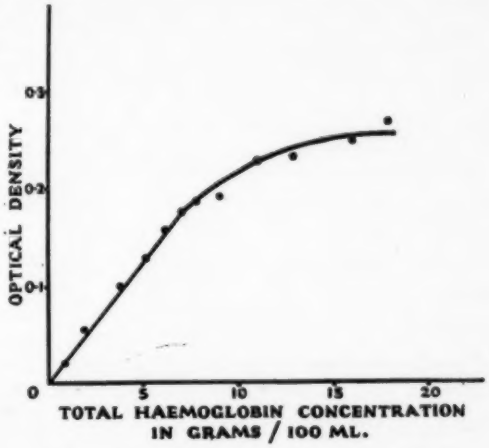

FIG. 2. *The optical densities of the diluted dye eluates obtained from the A haemoglobolin band, plotted against the original haemoglobin content of the haemolysates which were subjected to electrophoresis. Linearity can be seen to fall off at concentrations in excess of 7 g. of haemoglobin per 100 ml.*

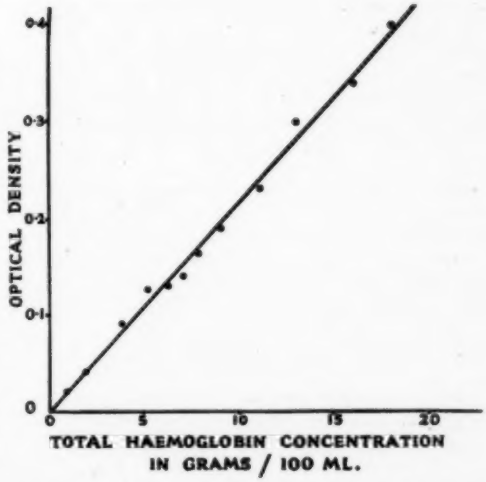

FIG. 3. *The optical densities of the diluted dye eluates obtained from the A_2 haemoglobin band, plotted against the original haemoglobin content of the haemolysates which were subjected to electrophoresis. Linearity holds throughout the range of concentrations used.*

ACCURACY An assessment of reproducibility was made by running at least three samples from each of two normal controls and two cases of thalassaemia on five successive days. Running, staining, and elution were all carried out in the routine manner, and the percentage A_2 of the total haemoglobin estimated. The figures obtained are presented in Table I.

TABLE I

VALUES FOR A_2 AS PERCENTAGE OF TOTAL HAEMOGLOBIN CONTENT FROM TWO NORMAL AND TWO THALASSAEMIC SUBJECTS WITH MULTIPLE ESTIMATIONS ON FIVE SUCCESSIVE DAYS

Type of Subject	*Total No. of Estimations*	*Mean Value for % A_2*	*Same Day*		*Different Days*	
			S.D.	*% Error*	*S.D.*	*% Error*
Normal	69	3·3	0·28	8	0·39	12
Thalassaemic	30	6·4		4		6

It can be seen from these data that the percentage error did not vary greatly between the normal and the thalassaemic patients, but that there was a significantly greater variation between runs on different days than between repeated estimations in the same run. No evidence could be found of a systematic day effect common to all the patients, and the extra variability between runs must therefore be attributable to variations in the degree of separation between the A and A_2 components and possibly to some variation in the cutting between the two bands.

In the evaluation of these results it should be borne in mind that the absolute quantity of the A_2 component is very small, being of the order of 50 μg. of protein. It can be deduced from the standard deviation on repeated runs that two-thirds of all results should be within one standard deviation, *i.e.*, $\pm 0{\cdot}4\%$ of the true value.

NORMAL RANGES Haemolysates from pregnant patients of northern European, Greek, and Italian extraction have been examined. In those patients classified as normal no evidence has been found of any haemoglobinopathy. The 24 patients, in whom the diagnosis of thalassaemia minor was made, include nine pregnant patients and 13 non-pregnant patients. Further details of these cases will be published at a later stage of these investigations. The values for these normal and abnormal groups of patients are given in Table II, and it can be seen that there is a significant separation between the normal and abnormal values ($P = 0{\cdot}001$).

TABLE II

NORMAL AND ABNORMAL VALUES FOR PERCENTAGE A_2 HAEMOGLOBIN

Type of Case	*Total No.*	*Quantity of A_2 as Percentage of Total Haemoglobin*		
		Mean	*Range*	*S.D.*
Normal pregnant patients of northern European origin	86	3·2	1·4-4·3	0·61
Normal pregnant patients of Mediterranean origin	115	3·2	1·4-4·3	0·64
Thalassaemia minor	24	7·0	5·1-8·6	1·20

DISCUSSION

At present it would appear that a raised A_2 value the most specific and sensitive single indicator of the thalassaemic trait; and although not pathognomonic in itself, raised values taken in conjunction with other peripheral blood findings may be regarded as virtually diagnostic. A requirement therefore exists for the development of a relatively simple and mass reproducible, yet reasonably accurate, test for the estimation of A_2 values. It appears that the described techniques amply fulfil the two former criteria, and that with carefully standardized conditions the accuracy is adequate. The major part of the experimental error probably derives from variations in the site of division between the two bands, but it can be anticipated that repeated estimation of any values, falling close to the upper range of normal, should eliminate the possibility of false results.

An advantage of dilution of the two components to similar optical densities is that they are within the range of linearity for the optical density of the dye. It is therefore possible to use the optical densities for calculations and to avoid the necessity for conversion into dye units.

The values for the normal range appear to be slightly higher than those obtained by Kunkel and Wallenius (1957) who found a mean value of 2·5% while estimating normals by the starch block method. The separation, however, between the normal and abnormal groups appears to be quite distinct.

We would like to thank the honorary medical staff of the Queen Victoria Maternity Hospital for making their cases available for investigation, and Dr. J. A. Bonnin, of the Division of Clinical Pathology, for his help and encouragement. We are also grateful to Dr. G. N. Wilkinson, of the Department of Mathematical Statistics, C.S.I.R.O., for statistical advice.

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The relationship to age and cerebral vascular accidents of fibrin and fibrinolytic activity

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SYNOPSIS Three 'normal' groups of people—young, middle-aged, and old—have been investigated with regard to the fibrin content and fibrinolytic activity of the blood. The fourth group consisted of middle-aged people who had previously sustained a cerebral vascular accident matched statistically for age with the middle-aged normals. It was concluded that fibrin increases with age but there is an interaction between age and sex, the female having a higher level in the young group and the male a higher level in the middle-aged group. There was no sex difference in the levels of fibrin in the old age group. Fibrinolytic activity increases with age and there is a positive correlation between fibrin and fibrinolytic activity but no age-sex interaction. Those with cerebral vascular accidents tended to have higher fibrin levels and lower fibrinolytic activity but the differences were not statistically significant. There did, however, appear to be an increase in antifibrinolytic activity in the cerebral vascular group.

There is in blood an enzyme system which is capable of digesting fibrin, and this property of blood is called fibrinolytic activity. Although a vast literature has gathered on the biochemical investigation of the component parts of this system, less attention has been paid to relating fibrinolytic activity to vascular disease.

An increase in antifibrinolytic activity has been found in certain cases of myocardial infarction (Guest, Daly, Ware, and Seegers, 1948) and correspondingly fibrinolytic activity has been found to be depressed (Hume, 1958a; Røjel, 1959). Intermittent claudication (Nestel, 1959) and arteriosclerosis in general have been reported to be associated with depressed fibrinolytic activity (Janiakowa, Kotlarek-Haus, Potoczek, and Tkaczewski, 1958).

As there is a possible relationship between fibrin formation and vascular disease, and since there is an increase in vascular disease with age, it seemed of interest to determine whether fibrinolytic activity varied with age and whether people with cerebral vascular accidents occurring in middle life differed from normal controls in this respect. In the process of this pilot study it was found that there were differences in the fibrin levels and these observations are also reported.

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MATERIALS AND METHODS

PATIENTS Group I consisted of 20 (seven men and 13 women) young healthy medical students and nurses whose ages ranged from 19 to 30 years.

Group II (10 men and 10 women) consisted of 10 healthy members of the hospital staff and 10 patients attending the Department of Psychological Medicine with minor psychiatric disturbances but not thought to have any organic or vascular disease. Their ages ranged from 40 to 65 years. Although not intensively studied to exclude occult vascular disease, it was felt that for a pilot study they were reasonably normal in this respect.

Group III consisted of 20 (four men and 16 women) old people whose ages ranged from 75 to 92 years. Their admission to hospital was for the following reasons: eight were social problems, seven had had a small cerebral episode resulting in weakness of one side, one had mild iron deficiency anaemia with urinary infection, one had glycosuria (no treatment required), one had mitral stenosis with cerebral embolus, one had leg pains of a vague nature, and one had diverticulosis. It was felt that although all had some evidence of arteriosclerosis, they differed from the 'normal' population in longevity, namely 67·3 years for males and 72·1 years for females at 1 year of age (Registrar-General for Scotland, 1960), and because of the mere fact of survival were likely to differ from people dying in middle life from vascular disease. No attempt was made to eliminate all the occult diseases to which the aged may well fall heir, *e.g.*, osteroporosis, chronic renal insufficiency, etc.

TABLE I

COMPOSITION OF GROUPS

Group I: Young Normals					Group II: Middle-aged Normals						Group III: Old Age						Group IV: Middle-aged Cerebral Cases					
No.	Age	Sex	Total Fibrin (mg.%)	Lysis in 1 Hour	No.	Age	Sex	Total Fibrin (mg.%)	Lysis in 1 Hour	Lysis in 2 Hours	No.	Age	Sex	Total Fibrin (mg.%)	Lysis in 1 Hour		No.	Age	Sex	Total Fibrin (mg.%)	Lysis in 1 Hour	Lysis in 2 Hours
1	30	M	291	52	1	40	M	309	64	206	1	85	F	379	272		1	50	M	206	0	86
2	27	M	202	77	2	48	M	270	0	0	2	80	F	515	193		2	32	M	245	0	168
3	21	M	189	88	3	50	M	218	12	102	3	79	M	180	51		3	62	F	386	129	386
4	25	M	231	64	4	44	F	245	0	0	4	84	F	322	13		4	49	F	386	51	51
5	25	M	167	38	5	48	F	180	0	26	5	87	F	309	309		5	53	F	245	0	81
6	29	M	232	52	6	46	F	193	0	0	6	84	F	206	52		6	59	M	309	129	309
7	24	M	193	26	7	53	F	296	39	103	7	79	F	245	0		7	53	F	257	25	39
8	23	F	180	13	8	53	M	463	0	0	8	78	F	329	0		8	64	M	296	0	77
9	20	F	347	167	9	60	M	373	141	219	9	75	F	322	116		9	44	M	193	0	0
10	19	F	270	13	10	50	F	296	51	78	10	78	F	386	77		10	54	F	270	0	25
11	19	F	212	0	11	54	M	360	231	360	11	76	M	202	0		11	56	M	412	0	245
12	21	F	218	0	12	58	M	386	77	141	12	92	M	399	399		12	55	M	412	0	0
13	25	F	257	0	13	61	F	218	38	154	13	87	F	270	25		13	65	F	566	154	489
14	28	F	360	51	14	47	F	218	51	64	14	82	M	450	38		14	54	M	245	13	52
15	24	F	296	0	15	47	F	180	13	64	15	82	F	386	13		15	63	F	438	78	168
16	24	F	218	51	16	64	F	502	180	257	16	85	F	438	26		16	56	M	579	64	257
17	22	F	296	39	17	55	F	245	0	65	17	85	F	386	0		17	48	M	283	65	154
18	23	F	335	117	18	58	M	605	0	0	18	81	F	438	39		18	49	M	399	167	399
19	23	F	386	129	19	65	M	463	154	283	19	81	F	309	309		19	58	F	218	0	89
20	25	F	193	26	20	58	M	309	64	180	20	79	F	482	77		20	54	F	438	103	193

Group IV consisted of 20 patients (11 men and nine women) whose ages ranged from 32 to 65 years and who had sustained a cerebral catastrophe resulting in hemiparesis or hemiplegia. Cerebral embolism was excluded as far as was possible but it was not possible to be certain whether cerebral thrombosis or haemorrhage had been the cause. Such an accident, however, was taken to indicate cerebral vascular disease. The illness had been established between five months and eight years with the exception of two patients who had developed paralysis three weeks previously. No patient was included whose cerebral vascular accident was of recent onset because it had been noted on two occasions that the fibrin levels were very high a few days after the incident, falling to normal levels in two weeks or so. A similar phenomenon has been reported after acute myocardial infarction (Gilchrist and Tulloch, 1952; Losner, Volk, and Wilensky, 1954) and it appears to be a usual occurrence after a variety of insults including surgical, chemical, and physical injuries as well as infections (Cohen, 1953).

Fibrinolytic activity was measured by the method of partial lysis previously described (Hume, 1958b). Briefly this involves measuring the amount of fibrin digested in a given period of time. Equal amounts of diluted plasma are added to test-tubes in a water-bath at 37°C. and clotted with thrombin. The amount of fibrin present after incubating for half an hour is taken as the initial level, and the difference in the fibrin content of the remaining tubes, estimated at hourly intervals, is a measure of the fibrinolytic activity. The method employed for estimating fibrin is a colorimetric one (Ratnoff and Menzies adapted by Holborn, 1951) and the results expressed as milligrams of fibrin per 100 ml. A low temperature technique was not employed. The blood was collected into graduated centrifuge tubes at room temperature and immediately spun in a M.S.E. super minor centrifuge at 4,500 r.p.m. for five minutes. The tubes were prepared and set up for incubation within 10 minutes after withdrawal of blood. The estimations were made between 1.30 p.m. and 2.15 p.m. in order to try and standardize the technique with regard to the effect of diet (Greig and Runde, 1957) and diurnal fluctuation (Buckell and Elliott, 1959).

RESULTS

AGE From a consideration of the ages in terms of groups (Tables I and II) it can be seen that there

TABLE II

MEAN AGE GROUPS STUDIED

Group	Male			Female		
	No. of Cases	Mean Age	S.D.	No. of Cases	Mean Age	S.D.
I	7	25·9	2·4	13	22·8	1·8
II	10	54·4	6·73	10	51·5	6·38
III	4	82·3	5·3	16	81·9	2·8
IV	11	51·5	8·4	9	56·8	4·9

is no statistical difference between groups II and IV, that is, the middle aged controls and the middle aged cerebral cases, nor is there any difference between the sexes in any group with regard to age, with the exception of group I where the sex difference is statistically significant ($P<0.01$).

FIBRIN Analysis of the initial fibrin levels in groups I, II, and III (Tables I and III) shows that there is a positive correlation between age and fibrin levels for both males and females (M.r. = +0·49 F.r. =

TABLE III

INITIAL MEAN FIBRIN LEVELS

Group	Male			Female			Male and Female		
	No. of Cases	Mean Fibrin Level (mg.%)	S.D.	No. of Cases	Mean Fibrin Level (mg.%)	S.D.	No. of Cases	Mean Fibrin Level (mg.%)	S.D.
I	7	215	37·8	13	274	65·5	20	254	67
II	10	375·6	106	10	257·3	91	20	316	115
III	4	307·8	118	16	357·6	82	20	348	111
IV	11	325·4	110	9	356	110	20	339	111

+0·47). This is illustrated in Fig. 1. However, analysis of variance yielded the fact that there was an interaction between sex and age. This was statistically significant ($P<0.01$) and is illustrated in Fig. 2. The sex differences analysed separately show that there is probably a significant difference between the means in group I ($P<0.05$) and similarly in group II ($P<0.05$). However, the results in group I are statistically suspect in the sex difference because there was a significant difference ($P<0.01$), as previously noted, between the ages of the males and females in this group. In actual fact, however, the age effect is so slight that the age difference could not explain the sex difference in this group. No significant difference was noted in group III. Although the mean fibrin level is much higher in the middle-aged females in group IV compared with that of the middle-aged females in group II (Table III), the difference between the means is not significant ($P-0.1$). Similarly there is no statistically significant difference between the means of the males in the same two groups.

LYSIS When lysis during the first hour is considered for groups I, II, and III (Tables I and IV) it can be seen that there is a positive correlation between age and lysis for both sexes (M.r. = +0·35 F.r. = +0·30) illustrated in Fig. 3. Analysis of variance revealed no sex and age interaction (Fig. 4). However, a variance ratio test shows that the difference in the variance between groups I and III is of high statistical significance ($P<0.001$), and between groups I and II and I and IV to be statistically significant ($P<0.01$). Analysis of the means revealed no statistically significant difference between the sexes in any group (Table IV), although the males had a higher mean reading and wider variation in group II. Although the mean lysis level is much lower in the middle-aged males in group IV as compared with the lysis level in the middle-aged males of group II, the differences were not statistically significant, nor was there any difference between the females of the same groups.

When lysis during the second hour was compared between groups II and IV (Table IV) again no statistically significant difference in variance or

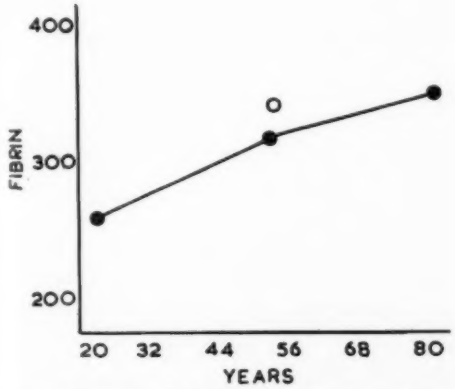

FIG. 1. *The mean fibrin levels (mg.%) for each group reflect an increase with years (O = mean fibrin level for cerebral cases).*

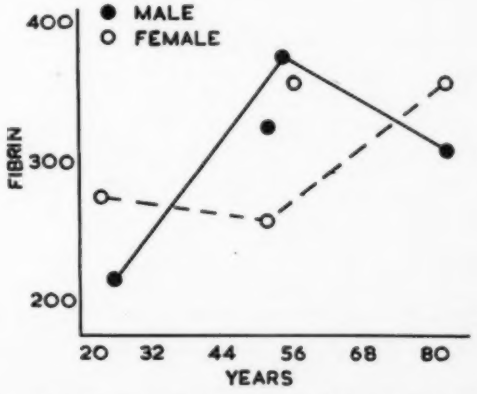

FIG. 2. *The mean fibrin levels (mg.%) for each sex shows an interaction between sex and age (● = male cerebral cases and O = female cerebral cases).*

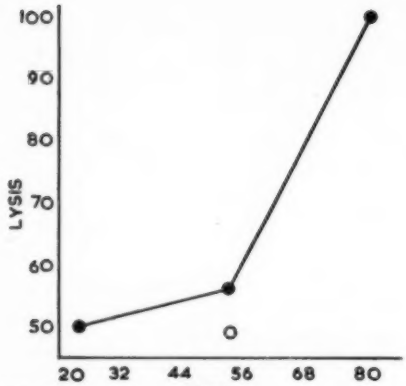

FIG. 3

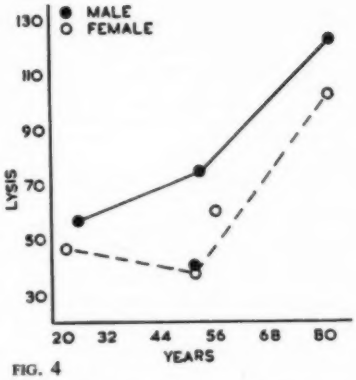

FIG. 4

FIG. 3. *Mean lysis (mg. fibrin digested %) for each group reflects an increase with years (O = mean lysis level for the cerebral cases).*

FIG. 4. *The mean lysis level (mg. of fibrin digested %) for each sex shows no sex-age interaction (● = male cerebral cases, O = female cerebral cases).*

means was detected. However, when the lysis was expressed as a percentage lysis in the second hour compared with the first hour, analysis of variance revealed that the difference between the two groups was probably significant ($P<0.05$) (Table IV), suggesting the presence of a retarding factor in group IV. The females in both groups showed a lower mean percentage but this was not statistically significant.

Consideration of fibrin levels and lysis together revealed a positive correlation between these two factors for both sexes (M.r. = +0·20 F.r. = +0·44).

DISCUSSION

Analysis of the initial fibrin levels in the first three groups reveals the fact that the fibrin content of blood increases with age. However, when the sexes are analysed separately it can be seen that there is an interaction between age and sex, the female having the higher level in the young age group and the male in the middle-aged group. In the old age group there is no sex difference. This suggests that the ageing process is associated with an increase in fibrin content of the blood and that it occurs at an

TABLE IV

MEAN LYSIS LEVELS FOR TWO PERIODS OF STUDY AND PERCENTAGE LYSIS COMPARED FOR EACH PERIOD

Group	*Male*			*Female*			*Male and Female*		
	No. of Cases	*Lysis in First Hour (mg.%)*	*S.D.*	*No. of Cases*	*Lysis in First Hour (mg.%)*	*S.D.*	*No. of Cases*	*Lysis in First Hour (mg.%)*	*S.D.*
I	7	56·7	20	13	46·6	54	20	50	45
II	10	74·3	75	10	37·2	52	20	56	67
III	4	122	161	16	95·1	109	20	100	121
IV	11	39·8	56·9	9	60	55·8	20	49	57
Group	*No. of Cases*	*Lysis in Second Hour (mg.%)*	*S.D.*	*No. of Cases*	*Lysis in Second Hour (mg.%)*	*S.D.*	*No. of Cases*	*Lysis in Second Hour (mg.%)*	*S.D.*
II	10	149·1	119	10	81·1	73·4	20	115	104
IV	11	158·8	125	9	169	155	20	163	139
Group	*No. of Cases*	*Change in % Lysis*	*S.D.*	*No. of Cases*	*Change in % Lysis*	*S.D.*	*No. of Cases*	*Change in % Lysis*	*S.D.*
II	10	14·3	17·3	10	9·1	18·7	20	11·7	18·1
IV	11	33·4	23·9	9	18·2	26·7	20	26·6	26·4

earlier age in the male. An increase in fibrin with age has previously been described (Schultz, 1956). It is of interest to speculate on whether the high fibrin levels in middle-aged males contribute to vascular disease in this group, especially in view of the fibrin theory of the genesis of atheroma (Duguid, 1954) and as the number of deaths from myocardial infarction between the ages of 45 and 49 years is five times greater in males (Registrar-General for Scotland, 1960). When the middle-aged cerebral cases are considered, it can be seen that there is a tendency for them to have higher fibrin levels and this is especially so of the females, but the differences are not statistically significant. However, the changes are such as to merit a more intensive study of this group.

Consideration of the fibrinolytic activity shows that once more there is an increase with age and also a positive correlation with fibrin levels. There is, however, within the limits of this pilot study, no interaction between sex and age and the differences are not statistically significant in any group between the sexes with regard to fibrinolytic activity. In view of the higher fibrin levels in the males compared with the females in middle life, it could be argued that the female has relatively more fibrinolytic activity with respect to fibrin than the male and possibly less susceptibility to thrombosis as a consequence of a greater clot-dissolving potentiality. It is interesting to note too that the lytic activity of the cerebral group as a whole is less than the controls and this applies especially to the males. The differences are not statistically significant but worthy of further investigation.

Finally, although the middle-aged cerebral group have only a relatively higher fibrin level and relatively lower fibrinolytic activity than their controls, when lysis during the second hour is expressed as a percentage of that of the first hour, it becomes apparent that the activity of the cerebral group has increased disproportionately. This could be explained by the presence of enhanced fibrinolytic inhibitor in those patients who have had cerebral vascular accidents. Antifibrinolytic activity has been described in cases of myocardial infarction (Guest *et al.*, 1948).

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The occurrence of post-gamma protein in urine: a new protein abnormality[1]

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SYNOPSIS As a result of studying the urine proteins of 223 individuals by starch gel electrophoresis, a new urine protein fraction has been recognized. On starch gel electrophoresis at alkaline *p*H the new fraction moves to a position appreciably nearer the cathode than the slowest-moving γ-globulin in normal serum. A small amount of this post-γ protein was found in the urine of 46 patients with clinical proteinuria, including 19 with the Fanconi syndrome and nine with multiple myeloma.

The nature, origin, and clinical significance of post-γ protein is discussed.

The proteins that occur in urine have received much less attention than those in serum, probably because the time-consuming step of preliminary concentration is usually necessary for their study. Even so there is a considerable literature dealing with the electrophoresis of urinary proteins in man (Longsworth and MacInnes, 1940; Luetscher, 1940; Gutman, Moore, Gutman, McClellan, and Kabat, 1941; Blackman and Davis, 1943; Moore, Kabat, and Gutman, 1943; Routh, Knapp, and Kobayashi, 1948; Farnsworth and Ruppenthal, 1951; Macheboeuf, Rebeyrotte, and Brunerie, 1951; Parviainen, Soiva, and Ehrnrooth, 1951; Rigas and Heller, 1951; Rundles, Cooper, and Willett, 1951; Rifkin and Petermann, 1952; Hauser, 1953; Jahnke and Scholtan, 1953; Schmidt, 1953; Slater and Kunkel, 1953; Soulier, 1953; Suenderhauf and Wunderly, 1953; Wiedermann, 1953; Boyce, Garvey, and Norfleet, 1954; Feruglio and Rimini, 1954; Wunderly and Caspani, 1954; Alvarez and de la Gandara, 1955; Del Bianco, 1955; Moeller and Steger, 1955; Osserman and Lawlor, 1955; Prill and Steger, 1955; Löwgren, 1956; Sellers and Marmorston, 1956; Engle, Woods, and Pert, 1957; Jim, 1957; Owen and Rider, 1957; Wolvius and Verschure, 1957; Angeli, 1958; Broch and Brodwall, 1958; Flynn and Stow, 1958; Hartmann, Lagrue, and Moretti, 1958; Heremans, 1958; Nedbal and Seliger, 1958; Schneiderbaur and Rettenbacher, 1958; Webb, Rose, and Sehon, 1958; Biserte, Breton, and Havez, 1959; Cirla, Salteri, and Bonomo, 1959; Hartmann, Lagrue, Raoult, Binet, and Milliex, 1959; Riva, 1959; Salteri, Cirla, and Bonomo, 1959). These studies have established that in all the conditions usually associated with proteinuria the lower molecular weight plasma proteins escape into the urine preferentially; thus albumin is predominant and the proportions of α_1-globulin and the non-lipid β-globulin are relatively high. In multiple myeloma the low molecular-weight Bence-Jones protein usually predominates, producing a peak somewhere between the α_2- and γ-globulin positions.

In 1958, Butler and Flynn reported that the slight proteinuria accompanying renal tubular disorders was often distinctive, being characterized on paper electrophoresis by the presence of a high proportion of α_2-globulin. With the object of defining this type of proteinuria more specifically it was decided to study the urinary proteins from cases of the Fanconi syndrome and related disorders by starch gel electrophoresis. With this technique it was found that the large α_2-globulin component on paper corresponded to fractions migrating between the β-globulin and albumin positions on starch gel. In addition it was found that many of the specimens contained a small amount of a protein which migrated more slowly than the slowest γ-globulin detectable in normal serum. The present communication is concerned with the occurrence of this previously undescribed post-γ protein.

MATERIAL AND METHODS

Fresh urine specimens and in many cases samples of clotted venous blood were obtained from 193 patients and relevant data concerning these is given in Table 1. For control purposes six individual samples were obtained

[1]Based on a paper read at the combined meeting of the Association of Clinical Pathologists and of the Association of Clinical Biochemists in London on 3 October 1959.

from healthy adults and pooled normal urines were collected from 12 adult males and from 12 adult females.

Single specimens of urine of about 200 ml. were examined in most instances, but the volume concentrated varied between 5 ml. and 3 litres. In 31 cases, including 20 where a post-γ protein was subsequently found in the urine, further specimens were obtained for confirmation of the findings. In all, 244 urine specimens were examined.

The urine specimens were filtered, the volume recorded, and the total protein measured by a modification of the turbidometric method of Kingsbury, Clark, Williams, and Post (1926). The urine proteins were subsequently concentrated, if possible to a level of approximately 2 to 3 g./100 ml., by one of two methods: the earlier samples were dialysed at 4°C. in Visking tubing against a saturated solution of gum acacia and the later specimens were ultrafiltered in similar tubing at room temperature as described by Grant (1957). Thiomersal B.P. was added as a preservative in the latter case, enough being added to give a final concentration of 10 mg./100 ml. The brown concentrate was centrifuged when a precipitate was present and the supernatant used for electrophoresis. The urine specimens were processed as swiftly as possible but if they had to be left at any stage they were stored at either 4°C. or at −20°C.

All the concentrates were examined by paper electrophoresis, using the qualitative technique of Flynn and de Mayo (1951), and by starch gel electrophoresis after the method of Smithies (1955). A few were also examined by electrophoresis on cellulose acetate strips using the method of Kohn (1957a). Two-dimensional electrophoresis, involving separation on cellulose acetate followed by fractionation on starch gel, was carried out according to the principles of the method of Poulik and Smithies (1958); tris-citrate/borate discontinuous buffer (Poulik, 1957) was used for the analysis in the second dimension.

In 12 instances immuno-electrophoresis was carried out. This was performed by diffusion into agar from strips cut from the starch gels, the detailed technique being similar to that of Kohn (1957b) for immunodiffusion from cellulose acetate strips. The antiserum used was a horse antihuman serum prepared by the Pasteur Institute.

In certain of the patients additional routine investigations were carried out, namely, microscopical examination of the centrifuged urine deposit and determination of the serum and urine calcium, plasma alkaline phosphatase, and blood urea.

RESULTS

Of the 244 urine specimens examined, 159 had protein detectable by the sulphosalicylic acid test. After concentration all had protein detectable on the starch gels and all but two, originally very dilute specimens, had protein demonstrable on the paper strips. The paper electrophoretic strips showed a whole spectrum of patterns, but four main categories could be recognized, examples of which are shown

TABLE I

CASES INVESTIGATED

	No. of Patients	*No. with Proteinuria*
Patients with Renal Tubular Dysfunction		
Fanconi syndrome		
Adult type	8	8
Acquired	7	6
Cystinosis	3	2
Dominant type	3	3
With cirrhosis but no cystinosis	1	0
Renal tubular rickets	5	0
Renal tubular acidosis	3	3
Hartnup disease	2	0
Cystinuria	2	2
Organic-aciduria syndrome	2	2
Atypical renal tubular defects	2	2
Wilson's disease	1	1
Severe potassium depletion	1	1
Sodium-losing nephritis	1	1
Pituitary diabetes insipidus	1	0
Galactosaemia	1	1
Patients with Conditions Commonly Associated with Proteinuria		
Urinary tract infections	9	8
Nephritis Type 2	6	6
Chronic nephritis	6	6
Diabetic nephropathy	5	5
Nephrosclerosis with malignant hypertension	4	4
Severe pre-eclamptic toxaemia	3	2
Disseminated lupus erythematosis	3	1
Congenital hypoplastic kidneys	2	2
Congestive cardiac failure	2	2
Nephritis Type 1	1	1
Polyarteritis	1	1
Orthostatic proteinuria	1	1
Patients with Neoplastic Disease		
Multiple myeloma	33	30
Carcinoma of bronchus	8	2
Hodgkin's disease	5	2
Carcinoma of breast	4	0
Carcinoma of bladder	3	3
Carcinoma of other sites	7	2
Lymphocytic lymphoma	1	0
Lymphatic leukaemia	1	0
Cordoma	1	0
Patients with Miscellaneous Other Conditions		
Sarcoidosis	7	2
Cirrhosis of liver	5	3
Idiopathic nephrocalcinosis	3	0
Aldrich's syndrome	2	0
Idiopathic steatorrhoea	2	0
Renal calculi	2	0
Idiopathic hypercalcuria	2	0
Hyperparathyroidism	1	0
Vitamin D intoxication	1	0
Idiopathic osteoporosis	1	0
Septic arthritis	1	0
Acute pancreatitis	1	1
Acute pericarditis	1	1
Bronchiectasis	1	0
Pulmonary tuberculosis	1	0
Tuberculous adenitis	1	0
Iron-deficiency anaemia	1	1
Duodenal diverticulae with megaloblastic anaemia	1	0
Thalassaemia	1	1
Sickle-cell anaemia	1	1
Obstructive jaundice	1	1
Purgative addict with potassium depletion	1	1
Recovering lead poisoning	1	0
Polymyositis	1	0
Hashimoto's thyroiditis	1	0
Von Gierke's disease	1	0
Pink disease	1	1
Analbuminaemia	1	0

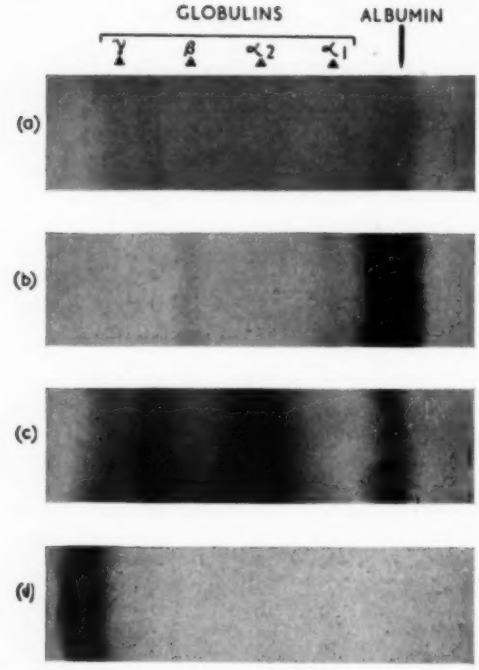

FIG. 1. *Examples of urine protein patterns on filter paper electrophoresis.*
(*a*) *Normal: urine from healthy adult.*
(*b*) *Glomerular proteinuria: urine from patient with Type 2 nephritis.*
(*c*) *Tubular proteinuria: urine from patient with organic-aciduria syndrome.*
(*d*) *Bence-Jones protein: urine from patient with multiple myeloma.*

in Fig. 1. The corresponding starch gel patterns are shown in Fig. 2. The starch gel patterns from the normal controls showed small amounts of albumin predominating over lesser amounts of a post-albumin fraction and traces of β and pre-albumin fractions. The 85 specimens from patients without clinical proteinuria were in most instances similar; seven, which were either exceptionally dilute or were concentrated less, showed only an albumin band, but others had in addition a trace of normal γ-globulin and a diffuse band in the slow α_2 region. The starch gel patterns of the 159 urines from patients with clinical proteinuria were more variable. Generally, those that had a glomerular leak type of pattern on paper electrophoresis showed a vast preponderance of albumin, a large amount of β-globulin, and lesser amounts of post-albumin, γ-globulin, and pre-albumin fractions. Those that had the tubular proteinuria pattern on paper showed a relatively small amount of albumin and a higher proportion of post-albumins and protein migrating between the β- and fast-α_2-globulin positions. Those with Bence-Jones protein usually showed a large proportion of one or two proteins situated in the γ, β, $\alpha\beta$, or slow α_2 region superimposed on an otherwise normal type of pattern. In two cases the Bence-Jones protein occupied a post-γ position. The interesting finding, however, was the detection of a trace quantity of a post-γ protein in some of the gel patterns from patients without myeloma. Such a post-γ protein is shown in Fig. 3. A total of 46

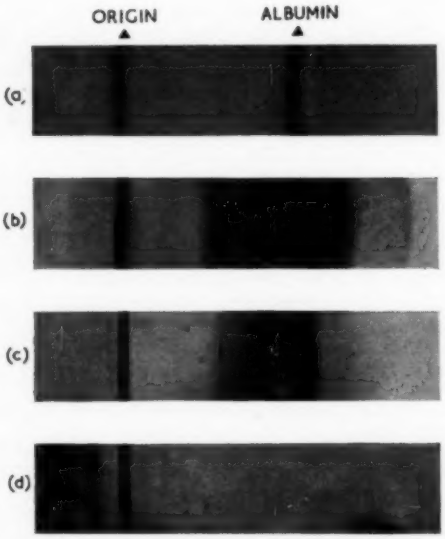

FIG. 2. *Examples of urine protein patterns on starch gel electrophoresis.*
(*a*) *Normal: urine from healthy adult.*
(*b*) *Glomerular proteinuria: urine from patient with Type 2 nephritis.*
(*c*) *Tubular proteinuria: urine from patient with acquired Fanconi syndrome.*
(*d*) *Bence-Jones protein: urine from patient with multiple myeloma.*

patients (22 female and 24 male) had a post-γ protein in the urine. In most instances this band was found at a distance from the insertion equal to twice that of the slowest-moving component of the normal γ-globulin, but in a few it occupied an intermediate position. In one of the patients a post-γ protein was also detected in the neat serum on one

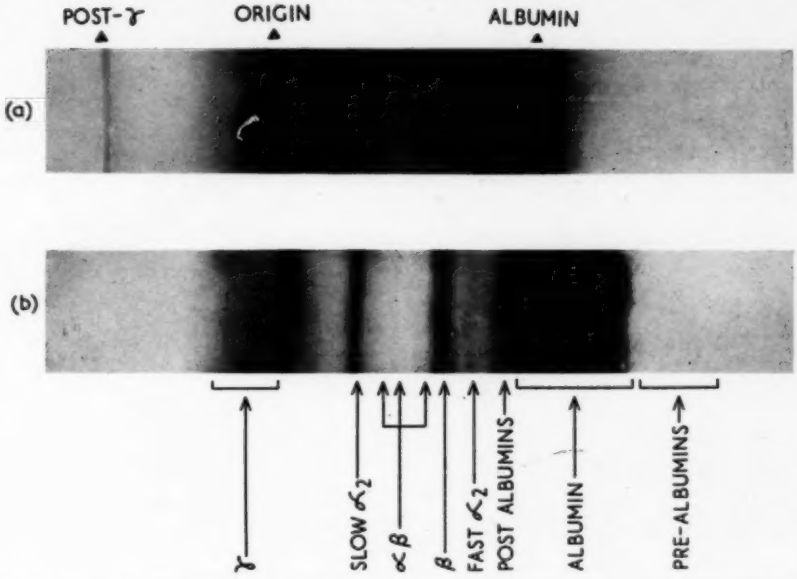

FIG. 3. *Urine showing post-γ protein on starch gel electrophoresis. (a) Urine from patient with adult Fanconi syndrome. (b) Serum from normal healthy adult.*

occasion. The diagnoses among the patients with a post-γ protein in the urine are shown in Table II. It is noteworthy that 27, *i.e.*, 79%, of the 34 patients who had renal tubular dysfunction with proteinuria, showed the post-γ band.

Electrophoresis on cellulose acetate of some of the urine concentrates showing a relatively high proportion of post-γ protein on the starch gels revealed the presence of a trace quantity of protein migrating more slowly than the slowest γ-globulin detectable in normal serum. Two-dimensional electrophoresis confirmed the identity of the post-γ fractions on cellulose acetate and starch gel.

TABLE II

PATIENTS WITH POST-γ PROTEIN IN THE URINE

	No. with Post-γ Band	*No. Examined*
Fanconi syndrome		
Adult type	8	8
Acquired	6	7
Cystinosis	2	3
Dominant type	3	3
Multiple myeloma	9	33
Renal tubular acidosis	3	3
Congenital hypoplastic kidneys	2	2
Atypical renal tubular defects	2	2
Organic-aciduria syndrome	2	2
Urinary tract infection	2	9
Sarcoidosis	2	7
Severe potassium depletion	1	2
Chronic nephritis	1	6
Hodgkin's disease	1	5
Primary biliary cirrhosis	1	1
Polyarteritis	1	1

The protein content and extent of concentration of the urines showing the post-γ protein is compared with similar data applicable to the remainder of the specimens in Fig. 4. This shows that the post-γ protein was usually found in the less marked proteinurias and that its detection was not simply a result of the urine having been concentrated to a greater extent.

The results of microscopy of the urine deposit in 60 cases indicated that there was no relation between the presence of a post-γ protein and the presence of red cells, pus cells, or casts. Attempts to correlate the presence of this protein with the level of the serum calcium, urine calcium, and plasma alkaline phosphatase were equally unsuccessful.

Examples of the results of immuno-diffusion from starch gel electrophoretic strips are shown in Fig. 5. This shows that in a urine with a marked post-γ protein there is some precipitation beyond the limit of the normal γ-globulin, but the centre of the arc does not correspond in position with the post-γ band. In the serum from the same patient, in which

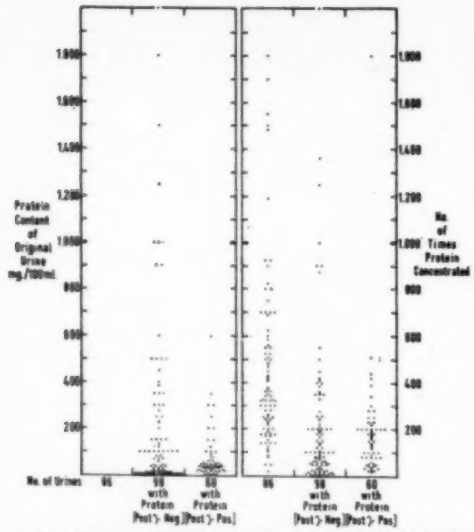
Protein Content of Original Urine mg./100ml.
No. of Times Protein Concentrated
No. of Urines
65
98 with Protein [Post-γ- Neg.]
60 with Protein [Post-γ- Pos.]

FIG. 4. *Diagram showing protein content of original urine specimens and extent of subsequent concentration process. (Median values for the six columns from left to right = 0, 65, 40, 333, 100, 155 respectively.)*

a post-γ band was discernible, a similar result is seen.

DISCUSSION

The finding of post-γ protein in the urine was quite unexpected and led us to consider whether it was an artefact, whether it was a minor component of normal urine, and whether it was derived from plasma or from the urinary tract.

The new fraction might have resulted from denaturation of the urine protein during the concentration process or during storage. Thiomersal cannot be incriminated as its use showed no correlation with the presence of the post-γ band. Similarly the extent and manner of the concentration process and the duration and conditions of storage bore no relation to its presence. Many of the specimens showing a post-γ fraction were in fact subjected to starch gel electrophoresis within 24 hours of collection and without having been frozen. The presence of unusual denaturation factors in the urine itself was excluded by showing that the starch gel pattern of normal serum was quite unaltered when it had been diluted with ultrafiltered urine from a patient with the adult Fanconi syndrome and recon-

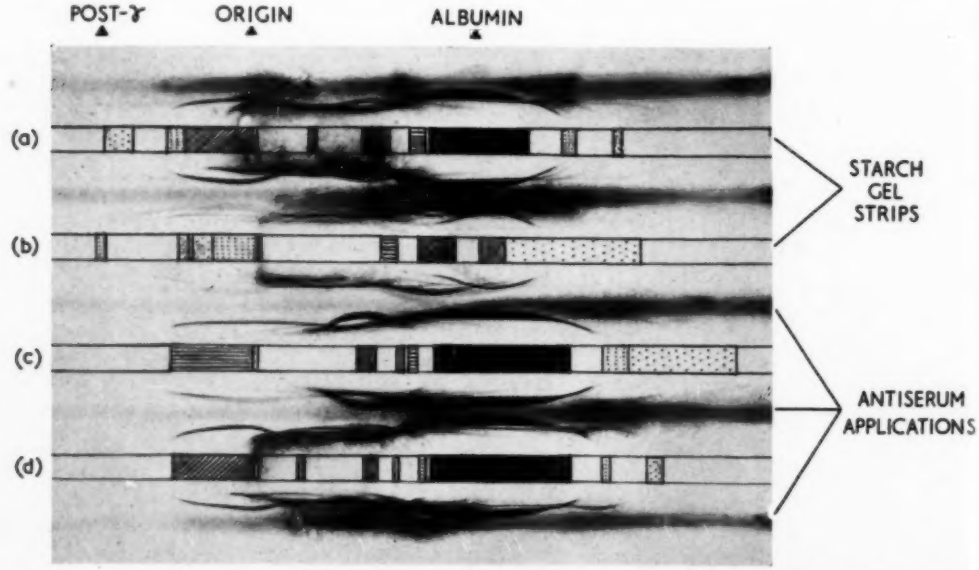

FIG. 5. *Immuno-electrophoretic precipitation patterns from starch gels using normal human serum antiserum. (a) Serum with post-γ protein: from patient with cystinosis. (b) Urine with post-γ protein: from patient with cystinosis. (c) Urine without post-γ protein: from patient with congestive cardiac failure. (d) Normal serum: from healthy adult.*

The position of the protein bands on the strips of starch gel is indicated in diagramatic form, correction having been made for the shrinkage that occurred on staining; the density of shading indicates the relative concentration of the protein fractions.

centrated exactly as a specimen of urine. The fact that the findings were reproducible on subsequent specimens also indicates that denaturation is an unlikely explanation.

The data concerning the degree of concentration of the urines (Fig. 4) show that the detection of a post-γ protein cannot be attributed simply to a greater degree of concentration of the specimens in question. It is clear, however, that this protein could easily have been missed if the process had not been carried far enough, as the amounts present were in all cases very small. The specimens of pooled normal urine were concentrated approximately 1,000-fold and yet no post-γ band was visible, so its detection at much lower degrees of concentration must be considered pathological.

Following the work of Grant (1957, 1959) on normal urine proteins, consideration was next given to whether this protein was a plasma protein that had reached the urine via the glomerulus, or whether it might be a protein that had been added to the urine from genito-urinary tract secretions or from breakdown of free cellular elements. Its occurrence in patients of both sexes and in infants of 3 and 7 months indicated that a genital origin was unlikely. Similarly, the absence of any relationship to the findings in the urine deposit made it improbable that it represented a breakdown product of red cells, pus cells, or casts. Free haemoglobin, detected by benzidine staining, was found in specimens both with and without post-γ protein, confirming that a red cell origin was unlikely. Immuno-electrophoresis failed to establish that the post-γ protein was derived from plasma; the immuno-precipitation patterns from the starch gels showed arcs extending beyond the normal γ region but these did not correspond with the post-γ band. In two of the patients with tubular disorders direct evidence in favour of a plasma origin for this protein was obtained; in one, with an unusually high concentration of post-γ protein in the urine, a post-γ protein was detected in the starch gel pattern of the neat serum, while in the other such a protein was demonstrated after concentrating the serum by freeze drying. In neither case did the post-γ band in the serum move as far back towards the cathode as in the urine, but the higher concentration of normal γ-globulin in the serum might well have influenced its migration in this way, just as one haemoglobin can influence the migration of another (Butler, Flynn, and Huehns, 1960). It seems likely, therefore, that post-γ protein comes from the blood.

Assuming that this protein leaks into the urine via the glomerulus, what is its origin and clinical significance? A review of the patients concerned revealed two points: that all were liable to have some disturbance of bone metabolism, and that nearly all had conditions that are occasionally, if not invariably, associated with renal tubular dysfunction. Evidence that this protein could be derived from bone was sought, but there was no correlation between its presence or absence and the results of calcium and alkaline phosphatase determinations. There was no relationship either with the administration of vitamin D or alkalis. To establish a possible association with renal tubular dysfunction, evidence of resorption defect was sought in some of those patients where it would have been unexpected—as in the two patients with sarcoidosis—but tests such as amino-acid chromatography showed no abnormality. All the patients who had the tubular proteinuria pattern had a post-γ fraction on starch gel but some patients with a glomerular proteinuria pattern had a similar band.

Other possibilities considered were that this protein might represent an unusual metal-protein complex or a genetically determined protein variant. The former was shown to be unlikely by an experiment in which the post-γ protein in a urine concentrate remained unaltered after dialysis against a large volume of buffer solution containing 0·6% ethylenediaminetetraacetic acid (Aronsson and Grönwall, 1957). As some of the patients with the post-γ protein were related, inheritance of a protein variant seemed a possibility. In three of the patients, however, one with potassium depletion, one with sarcoidosis, and one with a urinary tract infection, the post-γ protein disappeared from the urine as the patient recovered.

While the nature and origin of most of the post-γ globulins remain uncertain, in at least two of the patients with multiple myeloma the findings on paper electrophoresis taken in conjunction with the result of the heat test made it certain that the post-γ globulin was in fact a Bence-Jones protein. It may well have represented a minor Bence-Jones component in five of the remaining seven cases of myeloma with a post-γ band. Possibly the post-γ fraction represented an abnormal protein in all the cases, but the fact that it did not react with the antiserum to normal serum proteins cannot be accepted as proof. It seems reasonable to assume that the post-γ fraction represented a similar protein in the different patients, for while the mobility and compactness of the post-γ protein were variable, when two of the urines with apparently different post-γ proteins were mixed only a single band was obtained. Such differences as we have found might be due to the variation in concentration of normal γ-globulins.

Whatever its nature and origin, the post-γ protein is associated most frequently with conditions dis-

playing gross forms of renal tubular dysfunction. Our results do not permit us to give an estimate of its frequency, for although we have studied almost the complete spectrum of conditions associated with proteinuria, an undue proportion of patients with malignant disease and renal tubular disorders have been included as our ideas as to its aetiology have developed. Further, we may well have missed it in some of the urine specimens because of inadequate concentration. It seems likely, however, that its occurrence is relatively rare.

We wish to thank the physicians at University College Hospital, particularly Professor C. E. Dent, for providing us with most of the specimens, and also Sir Wilfred Sheldon and Drs. J. R. Nassim, S. W. Stanbury, J. M. Stowers, and J. M. Walshe for sending us specimens from patients with rare conditions. We also thank Dr. E. R. Huehns for help with the cellulose acetate electrophoresis, Mr. V. K. Asta for the diagram, and Mr. A. C. Lees for the photographs.

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ADDENDUM

Since submitting this paper for publication, Dr. M. Piscator, of the Institute of Hygiene, Karolinska Institute, Stockholm, has kindly provided us with the opportunity of examining the urinary proteins from two cases of chronic cadmium poisoning. In both instances a post-γ protein was detected.

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Physico-chemical and immunological observations on the abnormal proteins in three patients with cryoglobulinaemia

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With the technical assistance of
P. RATCLIFF

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SYNOPSIS Three new cases of cryoglobulinaemia are described, and in each case the presence of the cryoglobulin appeared to be unrelated to the clinical manifestations. Although when examined by paper electrophoresis the abnormal proteins behaved uniformly as normal gamma globulins, marked differences in nature were revealed when ultracentrifugal and immunological analyses were made. One patient had an abnormal protein which behaved both as a cryoglobulin and as a macroglobulin.

Abnormalities in the serum proteins may be quantitative or qualitative in nature. In the latter instance, certain globulins normally absent, or possibly present in trace amounts, are found in high concentrations. This group of protein disorders includes the myeloma proteins, macroglobulins, and cryoglobulins. The present paper is concerned with this last type of protein and three new cases are described.

The term cryoglobulin was introduced by Lerner and Watson (1947) to describe any protein which precipitated from whole serum on cooling and redissolved on subsequent warming. These writers described such an occurrence in the serum from a patient with purpura and referred to nine cases previously reported in the literature. It appears that Wintrobe and Buell (1933) first observed this phenomenon in a case of myelomatosis. Cryoglobulins have been found in a wide variety of pathological conditions: liver disease (Atlas, Cardon, and Bunata, 1943), kala azar, bacterial endocarditis, malaria, and chronic nephritis (Wertheimer and Stein, 1944).

Shapiro and Wertheimer (1946) first described cryoglobulinaemia in a patient suffering from polyarteritis nodosa. Lepow, Rubenstein, Woll, and Greisman (1949) described two further cases, and more recently Dreyfuss (1953) observed cryoglobulins in two cases of polyarteritis nodosa, whilst Butler and Palmer (1955) described a further case. One of the present patients suffered from polyarteritis nodosa.

For a detailed historical review, the reader is referred to the paper by Mackay, Eriksen, Motulksy, and Volwiler (1956) in which proved published cases of cryoglobulinaemia, up to that time, are analysed, 21 occurring in multiple myelomatosis and 32 in miscellaneous disorders.

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CASE REPORTS

CASE 1: CRYOGLOBULINAEMIA IN A CASE OF POLYARTERITIS NODOSA A 22-year-old woman was admitted to the Queen Elizabeth Hospital, Birmingham, under the care of Dr. C. F. Hawkins, in November 1956, with a three-week history of headache, vomiting, and pyrexia. The urine contained protein, red cells, and granular casts. It was thought initially that the patient was suffering from acute nephritis and during the course of an examination of the serum for complement activity, one of us noted the presence of a cryoglobulin. Splenomegaly and bilateral pleural effusions subsequently developed, the patient became uraemic and died as a result of a pulmonary embolus. Post-mortem (Dr. P. V. Best) and histological examinations confirmed the diagnosis of polyarteritis nodosa. Typical lesions were present in the liver, kidneys, and suprarenals. In addition there was a single encysted *Trichinella spiralis* larva in the tongue.

CASE 2: CRYOGLOBULINAEMIA IN A CASE OF STEATORRHOEA A 63-year-old man was admitted to the General Hospital, Birmingham, under the care of Dr. W. T. Cooke, in February 1958. The patient had been first seen in 1957

with a history of recurrent abdominal pain for many years. A laparotomy undertaken elsewhere in 1952 failed to reveal a cause for the symptoms. He had suffered from intermittent diarrhoea and the stools had been pale on occasions. He had an anaemia (haemoglobin 8·7 g. per 100 ml.; 100% = 14·8 g.) and a granulocytic leucopenia (total white blood cell count 4,800 per c. mm., polymorphonuclear leucocytes 7%).

At the time of admission the blood findings were confirmed and a marrow biopsy revealed a normoblastic erythropoiesis. The marrow contained a large number of plasma cells (30%). There was no evidence of a myeloma type protein in the serum or urine, but a cryoglobulin was detected in the serum by the biochemist, Dr. B. Northam, during a routine examination. Radiological examination of the intestinal tract and bones revealed no abnormality. Examination of the stools revealed a high fat content, and over a six-day balance study between 7·8 and 15·7 g. fat, with an average of 11·9 g., was being excreted per 24 hours. This is well above the normal upper limit of 6 g. per 24 hours (Cooke and French, 1958).

The possibility that this patient was suffering from cirrhosis was excluded by a liver biopsy which showed only mild fatty change. The Wassermann reaction was negative. A congo red test for amyloidosis also gave a negative result.

This patient was treated with A.C.T.H. and steroids have been continued since. There has been no change in the degree of cryoglobulinaemia or any significant improvement in the blood count, the total white cells varying between 2,000 and 3,800 per c. mm., with few polymorphonuclear leucocytes. This patient undoubtedly has steatorrhoea, but the significance of the raised plasma cell count in the marrow remains uncertain.

CASE 3: CRYOGLOBULINAEMIA IN A CASE OF LIVER DISEASE A 32-year-old male Pakistani was admitted to the General Hospital, Birmingham, under the care of Dr. P. Davison in May 1958, complaining of abdominal pain, weight loss, and fever. He had marked hepatosplenomegaly but no ascites. One of us detected a cryoglobulin in the serum while carrying out a complement estimation. Splenectomy was performed for hypersplenism, and the patient died following the development of a subphrenic abscess. The spleen weighed 2,025 g. Post-mortem and histological examinations (Dr. D. B. Brewer) indicated that cirrhosis of the liver was present, and the enlarged spleen had the changes associated with chronic congestion. There was no evidence of amyloidosis.

MATERIALS AND METHODS

Blood was collected in a warmed syringe from Cases 1, 2, and 3. In addition a larger volume was obtained from the splenectomy specimen in Case 3.

Cryoglobulins were isolated by centrifugation of sera cooled to 4°C. and then dissolved in 0·85% saline at 37°C. Purification was attempted by repeating this process several times.

ESTIMATION OF SERUM PROTEINS BY PAPER ELECTROPHORESIS Total serum proteins were estimated by a Biuret method, and individual components were separated and studied quantitatively by the paper electrophoresis technique described by Hardwicke (1954). Cryoglobulin concentrations were estimated from the differences in protein concentration of serum at 37°C. and at 4°C.

ULTRACENTRIFUGAL ANALYSIS Whole sera and isolated cryoglobulins were analysed at 60,000 r.p.m. and 20°C. in a Spinco model E machine incorporating a Philpott-Svensson optical system. Specimens were diluted to contain approximately 1·0 g. protein per 100 ml. and dialysed for 16 hours against 1 litre of acetate buffer (*p*H 4·7; I = 0·10), containing 0·2M. NaCl, since cryoglobulins have been reported to be more soluble at low temperatures in this medium (Gunz, 1956). Relative concentrations of the various ultracentrifugal components were estimated from the enlarged tracings of the Schlieren patterns.

IMMUNOLOGICAL STUDIES Serum complement estimations were made as previously described (Walton and Ellis, 1958), using fresh sera.

Gel-diffusion precipitin analyses were carried out with rabbit antisera raised against Freund adjuvants of the cryoglobulins from Cases 2 and 3, injected subcutaneously.

Immuno-electrophoretic analyses (Grabar and Williams, 1955) were carried out using specific antisera against normal human γ-globulin, as well as against two of the cryoglobulins.

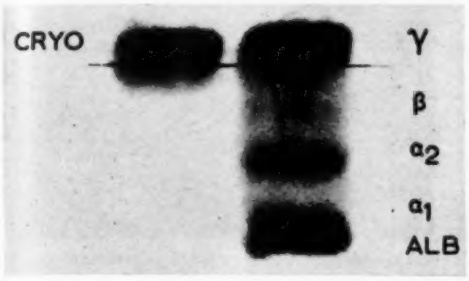
RESULTS

In each instance the cryoglobulin precipitated out from the whole serum on cooling as a white flocculent precipitate. This process was reversible, the precipitate readily going into solution on warming to 37°C. The temperature at which precipitation occurred was between 28°C. and 32°C. in the case of the cryoglobulins from Cases 1 and 2, whereas that from Case 3 was only precipitated after the serum had been allowed to stand at 4°C. overnight.

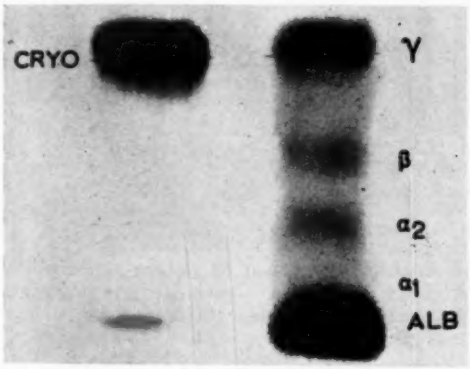
Microscopical examination indicated the amorphous nature of the precipitate, and whether the precipitate was allowed to form slowly or rapidly there was no evidence of crystal formation, which was described in a case by Shapiro and Wertheimer (1946).

PAPER ELECTROPHORESIS STUDIES Paper electrophoresis analyses of sera (Table I and Fig. 1) indicated that all three cryoglobulins were contained in the gamma globulin fraction. The cryoglobulin preparations obtained by cooling the sera were found, after purification, to be electrophoretically homogeneous, except for the presence of a trace of albumin in that from Case 2.

ULTRACENTRIFUGAL ANALYSES Although paper electrophoresis studies showed an apparent uniformity



1a



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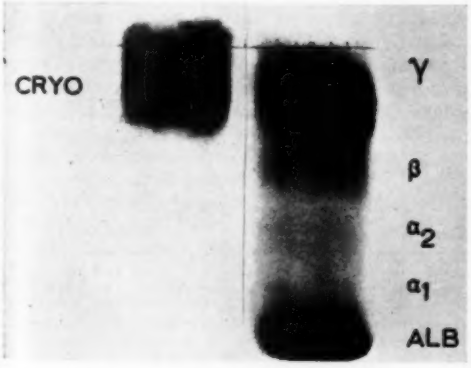

1c

FIG. 1. (a) *Case 1*, (b) *Case 2*, (c) *Case 3. Results of paper electrophoretic analyses of whole serum and isolated cryoglobulins from the three cases. (Alb = Albumin; α_1,α_2, β, and γ = normal globulin components; CRYO = cryoglobulin).*

TABLE I

PAPER ELECTROPHORETIC ANALYSES OF SERA FROM 3 CASES OF CRYOGLOBULINAEMIA

Case	Concentrations of Protein Components (g.%)					
	Albumin	α^1	α^2	β	*Cryoglobulin*[1] $+\gamma$	*Cryoglobulin (estimated)*
1	2·12	0·39	1·23	0·70	2·90	0·75
2	3·03	0·52	0·86	0·87	1·83	1·15
3	2·36	0·44	0·50	0·80	3·20	0·23

(The value for the cryoglobulin concentration was estimated from the difference in protein concentration of the serum before and after removal of the cryoglobulin.)

of the cryoglobulins, examination in the ultracentrifuge revealed marked differences. Only the preparation from Case 2 was found to possess a similar sedimentation coefficient (7S) to that of normal serum gamma globulin. As shown (Table II), one of the other preparations (Case 1) appeared to be composed entirely of lower molecular weight material (4·6S), whereas the third (Case 3) was largely composed of higher molecular weight protein (19S and 22S).

The ultracentrifugal patterns obtained (Fig. 2) indicate that, apart from the small amount of 22S material detected in the cryoglobulin from Case 3, all the isolated proteins were composed of normal serum ultracentrifugal components. Contamination with non-cryoglobulin constituents (to the extent of 10 to 25% of the total protein concentration) seemed probable in the cryoglobulin preparations from the sera of Cases 2 and 3. The low concentration of protein in the solution of the cryoglobulin preparation from Case 1 diminished the chance of detecting similar contamination. Moreover, this specimen had been stored for several weeks at a low *p*H (acetate buffer *p*H 4·5) before ultracentrifugal analysis, and it is possible that the cryoglobulin had been degraded. The sedimentation coefficient could not be determined with any high degree of accuracy because of the difficulty in establishing the mid-point of the very small Schlieren peak. It seems possible, therefore, that the cryoglobulin from Case 1 was originally associated with the abnormally high concentration of 7S component found in the whole serum.

SERUM COMPLEMENT Serum complement was normal in the sera from Cases 2 and 3 and no anticomplementary activity could be detected. In contrast there was a marked depression in serum complement activity in the whole serum from Case 1. There was a rise in activity in the serum after the cryoglobulin had been removed by centrifugation at 4°C., and the washed cryoglobulin produced a depression of

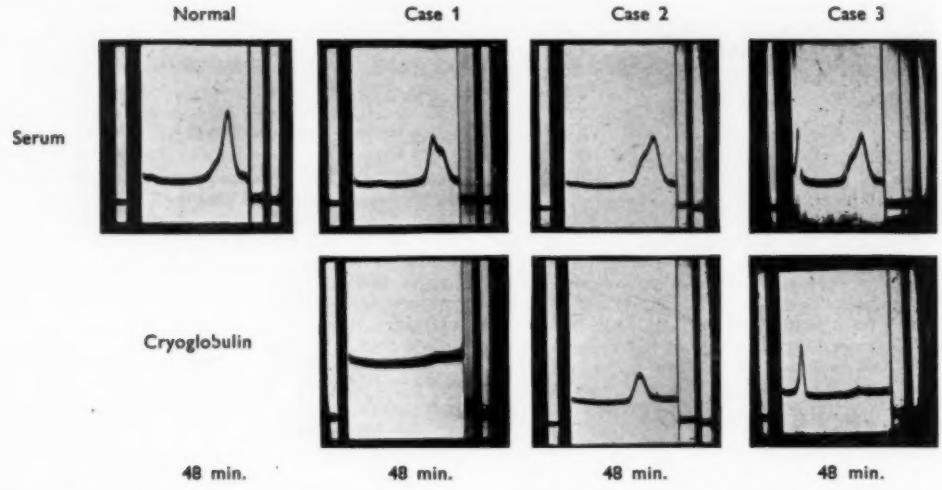

FIG. 2. *Ultracentrifugal patterns of whole sera and isolated cryoglobulins. Representative selected patterns at 48 minutes in each case are shown and compared with the normal.*

activity when added to normal serum. The residual serum after removal of the cryoglobulin still possessed some anticomplementary activity. It is concluded that the anticomplementary activity resided in part in the cryoglobulin component.

PRECIPITIN REACTIONS AND IMMUNOELECTROPHORESIS STUDIES The cryoglobulin from Case 1 cross-reacted with an antiserum to normal 7S gamma globulin. Immunoelectrophoretically, however, an abnormally shaped precipitin line was obtained (Fig. 3).

The serum from Case 2, when examined by immunoelectrophoresis (Fig. 4), gave a normal and an abnormal gamma globulin precipitin line with an antiserum to normal 7S gamma globulin. The isolated cryoglobulin behaved immunoelectrophoretically in a similar manner to that from Case 1.

Comparative analysis of the cryoglobulin from Case 2 and normal 7S gamma globulin by the Ouchterlony gel-diffusion precipitin technique, using a monospecific antiserum to normal 7S gamma globulin, was carried out. A spur formation of the precipitin line was obtained, (Korngold, 1956), suggesting that the cryoglobulin is deficient in one or more of the normal gamma globulin determinant groups and is behaving as an incomplete antigen.

TABLE II

RESULTS OF ULTRACENTRIFUGAL ANALYSES

Case	*Specimen*	*Total[1] Protein (g./100 ml.)*	*Concentration of Component (g./100 ml.)*									
			22S		*19S*		*10S*		*7S*		*4·5S*	
			$S_{20,w}$	*Concentration*	$S_{20,w}$	*Concentration*	$S_{20,w}$	*Concentration*	$S_{20,w}$	*Concentration*	$S_{20,w}$	*Concentration*
1	Serum	7·34			18·8	0·16			6·5	3·37	4·2	3·81
	Cryoglobulin solution	0·05									4·7	0·05
2	Serum	7·11			19·0	0·26			6·9	2·30	5·0	4·55
	Cryoglobulin solution	1·20							7·4	1·08	5·1	0·12
3	Serum	7·30	22·1	0·15	15·0[2]	1·70			6·5	1·88	4·2	3·57
	Cryoglobulin solution	1·00	25·5	0·05	17·4[2]	0·74	9·9	0·02	6·8	0·19		

[1]Allowance for dilution before ultracentrifugal analysis has been made in calculating the concentrations of the serum components, whereas the concentrations of the cryoglobulin preparations are those of the solutions anlysed.
[2]The apparently low values for the sedimentation coefficient of the 19S component in the serum and cryoglobulin from Case 3 reflect the marked concentration dependence of this material.

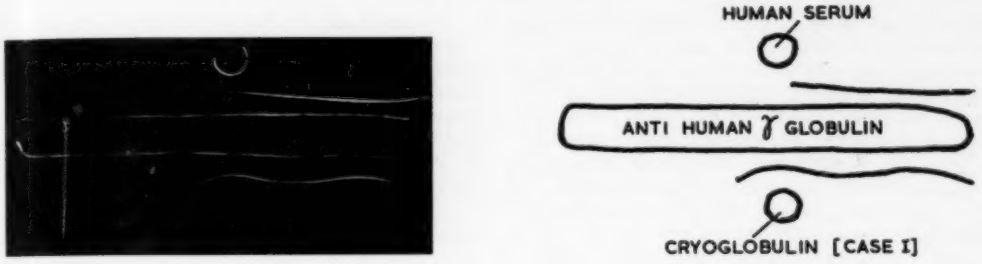

FIG. 3. *Immunoelectrophoresis of isolated cryoglobulin from Case 1.* (a) *Illustration of precipitin lines;* (b) *key. Note the abnormally shaped precipitin line, compared with that usually obtained.*

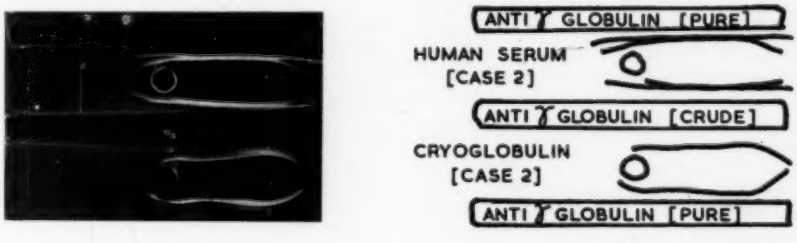

FIG. 4. *Immunoelectrophoresis of whole serum and isolated cryoglobulin from Case 2.* (a) *Illustration of precipitin lines;* (b) *key. Note the two precipitin lines in the γ region. One of these is abnormally situated, and appears in the cryoglobulin preparation.*

Cryoglobulin II gave no precipitin reaction when tested with the antiserum raised against the cryoglobulin III.

Gel-diffusion precipitin analyses using a specific antiserum against the cryoglobulin from Case 3 supported the physico-chemical evidence that the abnormal protein in this case was an 19S macroglobulin; no reaction was observed when the antiserum was tested either with normal 7S gamma globulin or with the cryoglobulin from Case 2.

DISCUSSION

Cryoglobulinaemia is not pathognomonic of any single disease entity and may be encountered in a number of diseases of different aetiology, as previously mentioned. In the present three cases the phenomenon was discovered incidentally while sera were being examined for another purpose. In each case there was no evidence to suggest that the cryoglobulinaemia was responsible for any of the clinical features. Thus the symptoms usually associated with the presence of cryoglobulins, such as cold-induced urticaria, and purpuric or thrombotic phenomena, such as digital necrosis or blindness (Hansen and Faber, 1947), were entirely absent. Deliberate attempts to induce cold urticaria by the application of ice to the forearm in Cases 2 and 3 were unsuccessful.

Muirhead, Montgomery, and Gordon (1952) described a case of pulmonary hypertension arising from thrombo-embolism of the pulmonary vessels due to cryoglobulinaemia, and were able to demonstrate histologically the abnormal protein within the thrombi. Histological examination of tissue obtained at necropsy from the present Cases 1 and 3 failed to reveal any convincing evidence of the intravascular deposition of the abnormal proteins.

The present three cases are examples of cryoglobulinaemia occurring secondary to some other recognizable disorder. This is the common type, and that described by Volpé, Bruce-Robertson, Fletcher, and Charles (1956), in which investigation failed to reveal an underlying cause, is relatively uncommon. From a perusal of case reports in the literature, it is clear that the actual concentration of cryoglobulin

in the serum bears no constant relation to the type or severity of the clinical manifestations; large amounts of the abnormal protein may be present in the absence of symptoms attributable to it, and conversely, marked symptoms may occur when only low concentrations of cryoglobulin are detectable.

It is of interest that one of the present patients was suffering from polyarteritis nodosa, since the association has previously been noted (see references above). We have examined the sera from two other cases of polyarteritis nodosa, with negative results.

The physico-chemical properties of the cryoglobulins appear to vary considerably. Although the present three cryoglobulins had similar electrophoretic mobilities, and behaved as γ globulins, the ultracentrifugal analysis revealed marked differences in their individual properties. It is usual for cryoglobulins to behave like normal serum γ globulin when examined by the technique of paper electrophoresis, although mobilities ranging from that of α_2 globulin to that of γ_2 globulin have been described (see Mackay *et al.*, 1956, for references). Ultracentrifugally, the cryoglobulin from Case 2 was indistinguishable from normal γ globulin, whereas that from Case 3 behaved as a macroglobulin. This phenomenon of an abnormal protein having the properties of a cryoglobulin and a macroglobulin has been observed previously, for example, by McFarlane, Dorey, Slack, and Papastamatis (1952) and by Petermann and Braunsteiner (1954) in the case of a cryoglobulin occurring in serum, and by Abrams, Cohen, and Meyer (1949) in the case of a cryoglobulin isolated from the lymph nodes removed from a patient suffering from lymphosarcoma.

It seems likely that the cryoglobulins are abnormal protein constituents of human serum, and do not represent an increase in the concentration of components normally present only in trace amounts. This appears to be supported by the results of the immunological analyses carried out on the present three cryoglobulins. Thus cryoglobulins in Cases 2 and 3 show immunological characteristics which readily distinguish them from each other and from normal 7S γ globulin.

This conclusion is consistent with the results of structural studies on abnormal human serum globulins carried out by Putnam (1958), who demonstrated that multiple myeloma globulins, macroglobulins, and cryoglobulins can be distinguished from each other and from normal γ globulin by their N-terminal residues, as well as by their physico-chemical properties.

We are indebted to Drs. W. T. Cooke, C. F. Hawkins, and P. Davison for permission to publish details concerning their patients, to Professor J. R. Squire for helpful criticism, and to Professor P. Gell for making available certain data concerning the immunological aspects of the study. One of us (H.A.E.) was in receipt of a grant from the United Birmingham Hospitals Endowment Fund when the study was undertaken.

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The effect of corticotrophin zinc on plasma 17-hydroxycorticoids as a test of adrenal cortical function

BY

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SYNOPSIS The response of plasma 17-hydroxycorticoids to corticotrophin has been determined using a simplified technique. Steroid levels appeared to be maximal four hours after the injection of 40 units of corticotrophin zinc. No response was obtained in cases of Addison's disease and a sluggish increase occurred in hypopituitarism. The effect of steroid therapy is discussed.

The response of 17, 21-dihydroxy-20-ketosteroids (17-hydroxycorticoids) in the plasma to the administration of corticotrophin provides a direct and sensitive measure of adrenal cortical function (Eik-Nes, Sandberg, Nelson, Tyler, and Samuels, 1954; Eik-Nes, Sandberg, Migeon, Tyler, and Samuels, 1955; Bayliss and Steinbeck, 1954). The widespread use of this procedure has been limited, however, by the technical complexity of the methods available for the estimation of plasma 17-hydroxycorticoids. The method of Nelson and Samuels (1952), which has been mostly favoured, involves the need for column chromatography with specially prepared florisil, making it unsuitable for ordinary laboratory use. A method which dispensed with the need for chromatography was subsequently devised by Silber and Porter (1954), and more recently Peterson, Karrer, and Guerra (1957) have further modified the Silber-Porter procedure, so that the estimation of plasma 17-hydroxycorticoids has now become greatly simplified.

For the adequate assessment of its function, prolonged stimulation of the adrenal cortex is necessary. An intravenous infusion of corticotrophin for six or eight hours is often used but this is inconvenient and is occasionally accompanied by hypersensitivity reactions, especially if given repeatedly to a patient suffering from adrenal insufficiency. Long-acting forms of corticotrophin for intramuscular use are easier to administer and are now more consistently reliable than formerly. Geller, Gabrilove, and Soffer (1957) and Jenkins (1958) have shown that a single injection of the aqueous corticotrophin zinc preparation is followed by a very prolonged increase in 17-hydroxycorticoid secretion. It seemed, therefore, that the estimation of plasma 17-hydroxycorticoid levels by the Peterson technique before and after the injection of corticotrophin zinc would provide a relatively simple test of adrenal cortical function.

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METHODS

Twenty-five millilitres of blood was taken from the patient between 9.30 and 10.0 a.m. and transferred to a bottle containing heparin. The plasma was separated immediately and frozen until required. An injection of 40 units of corticotrophin zinc was given intramuscularly and four hours later a further sample of blood was taken. In some cases the test was repeated on the following day.

ESTIMATION OF PLASMA 17-HYDROXYCORTICOIDS The method of Peterson *et al.* (1957) was used with slight modifications. This method involves the extraction of 5 ml. plasma with methylene chloride, washing the extract with dilute alkali, and shaking with the phenylhydrazine-sulphuric reagent of Porter and Silber (1950). Colour development is read at 410 mμ and suitable blanks are incorporated to allow for non-specific colour due to the acid alone. Steroids, other than hydrocortisone, having the 17, 21-dihydroxy-20-keto structure, are also estimated by this procedure but Peterson, Wyngaarden, Guerra, Brodie, and Bunim (1955) have shown, both by counter-current and paper chromatographic methods, that over 90% of the phenylhydrazine-reacting material is ordinarily hydrocortisone. Certain drugs, such as chloral, quinine, and paraldehyde, interfere with the colour reaction and should not be given before carrying out the test.

The following details refer to modifications of the original method. Methylene chloride was purified by

washing first with concentrated sulphuric acid, then with water, drying with anhydrous sodium sulphate, and distillation through a fractionating column. Instead of swirling the plasma with methylene chloride as originally described by Peterson, extraction was more conveniently performed by shaking it vigorously in a glass stoppered tube for five minutes; emulsions were easily broken by centrifugation. The methylene chloride extract was shaken with 0·6 ml. phenylhydrazine-sulphuric acid reagent, the methylene chloride was removed, and the reagent allowed to stand at room temperature overnight. The optical density was then read at 410 mμ in micro-cuvettes of 0·5 ml. capacity and 10 mm. light-path, using a Unicam S.P. 600 spectrophotometer, the carrier of which had been specially adapted. (An attachment enabling micro-cuvettes to be used with the S.P. 600 instrument is shortly to be made available by Unicam Instruments Ltd.)

The recovery of hydrocortisone added in amounts of 1·0 μg and 2·0 μg is shown in Table I.

TABLE I

RECOVERY OF HYDROCORTISONE ADDED TO PLASMA

17-Hydroxycorticoids (μg. per 5 ml. plasma)

Plasma	*Plasma + 1·0 μg. Hydrocortisone*	*Plasma + 2·0 μg. Hydrocortisone*
0·58	1·52	2·30
0·51	1·48	2·30
0·61	1·50	2·29
0·57	1·57	2·34
0·61	—	2·42
0·57	—	2·37
Mean 0·58	1·51	2·34
Mean recovery	0·93 (93%)	1·76 (88%)

RESULTS

NORMAL RESPONSE The figure shows the plasma 17-hydroxycorticoid levels at two-hourly intervals up to six or eight hours after the administration of 40 units of corticotrophin zinc to four normal individuals. The maximum increase occurred at four to six hours after the injection and in all subsequent tests the four-hour level was used.

The response of 20 patients without evidence of endocrine disease showed control levels which ranged from 5 μg. to 28 μg. per 100 ml. (mean 13 μg. S.D. ±6) and four hours after corticotrophin the values were 21 μg. to 53 μg. (mean 35 μg. S.D. ±8).

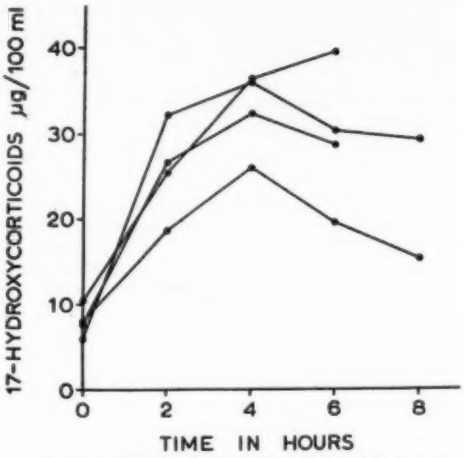

FIG. 1. *The effect of 40 units of corticotrophin zinc on the plasma 17-hydroxycorticoids in four normal persons.*

ADDISON'S DISEASE The application of this test to five patients suffering from Addison's disease is shown in Table II. In four cases the control values were low or absent and there was no significant response to corticotrophin. In case M.W. the clinical appearance was suggestive of Addison's disease, as shown by increased pigmentation and undue weakness, but the control level was normal. A lack of response to corticotrophin, however, which was confirmed by comparable urinary steroid findings, indicated a partial adrenal insufficiency. This condition has been described by Eik-Nes *et al.* (1955) and Abu Haydar, St. Marc, Reddy, Laidlaw, and

TABLE II

RESPONSE OF PLASMA 17-HYDROXYCORTICOIDS IN ADRENAL INSUFFICIENCY

Case	*Age*	*Sex*	*Diagnosis*	*Plasma 17-Hydroxycorticoids (μg. per 100 ml.)*		
				Control	*A.C.T.H. (1st day)*	*A.C.T.H (2nd day)*
F.D.	52	F	Addison's disease	4	0	0
A.P.	36	F	Addison's disease	3	1	4
E.G.	36	F	Addison's disease	0	4	
D.W.	47	F	Addison's disease	0	0	
M.W.	48	F	Partial Addison's disease	9	11	
R.C.	40	M	Hypopituitarism	2	14	26
H.S.	53	M	Hypopituitarism	0	7	15
A.S.	63	M	Hypopituitarism	0	11	16
A.H.	58	M	Hypopituitarism	0	3	15

Thorn (1958), and it appears that the deficient adrenal tissue is incapable of a response since it is already maximally stimulated by endogenous corticotrophin.

HYPOPITUITARISM Table II also shows the results obtained in four cases of hypopituitarism. These patients had all received either radiotherapy or surgical treatment for a pituitary tumour. Low or absent control values were again found but in contrast with the primary adrenal insufficiency of Addison's disease, an increase after corticotrophin was obtained, although this was not definitely apparent in every case until the second day of stimulation.

EFFECT OF STEROID THERAPY The well-known inhibitory effect of corticosteroid therapy on the adrenal cortex has been investigated (Table III). Tests were carried out on the day following discontinuation of the drug. The large doses of steroids administered to patients E.P. and F.S. resulted in complete adrenal inhibition, and 75 mg. of cortisone given over a longer period to W.B. also caused marked suppression. On the other hand, E.W., who was receiving 12·5 mg. of cortisone, after previously having been given much higher doses, now appeared to have normal adrenal function.

DISCUSSION

The results demonstrate that in the presence of normal adrenal function plasma 17-hydroxycorticoids were increased beyond doubt four hours after an intramuscular injection of corticotrophin zinc. The values obtained by means of the simplified procedure are comparable with those of Eik-Nes *et al.* (1954) and Bayliss and Steinbeck (1954) who used a more complicated technique for the estimation of plasma 17-hydroxycorticoids and an intravenous infusion of corticotrophin.

The lack of response in the primary adrenal failure of Addison's disease differentiates this condition from adrenal insufficiency secondary to hypopituitarism in which a somewhat sluggish increase in plasma steroids was obtained. Although a definite increase occurred on the second day of stimulation in the cases studied, it is conceivable that the response of some patients may be further delayed and an even longer period when corticotrophin is given may be required.

The adrenal insufficiency which accompanies steroid therapy is an important clinical problem. The results obtained in this investigation showed that adrenal suppression occurred when steroids equivalent to a daily dosage of 75 mg. or more of cortisone were given. Christy, Wallace, and Jailer (1956) have emphasized that after stopping the drug the length of time required before recovery depends upon the duration of therapy as well as the dosage, although considerable variation exists in individual patients. This aspect has not been studied in detail here, but one patient had recovered normal adrenal function within three weeks of reducing the dosage to 12·5 mg. of cortisone daily after receiving presumably suppressive doses for seven years. It should be remembered, however, that a normal response to exogenous corticotrophin does not necessarily imply complete recovery of the whole pituitary-adrenal system.

The use of plasma 17-hydroxycorticoid levels can give a more direct indication of adrenal cortical function than can be obtained from urinary 17-ketosteroid and 17-ketogenic steroid values, since the latter are largely a measure of steroid metabolites and may be affected by such extra-adrenal factors as hepatic or renal disease; in these conditions abnormally low values may be encountered when plasma levels remain within the normal range.

The method which is described above for the estimation of plasma 17-hydroxycorticoids is reliable

TABLE III

RESPONSE OF PLASMA 17-HYDROXYCORTICOIDS AFTER STEROID THERAPY

Case	*Age*	*Sex*	*Diagnosis*	*Steroid Therapy*	*Plasma 17-Hydroxycorticoids (μg. per 100 ml.)*		
					Control	*A.C.T.H. (1st day)*	*A.C.T.H. (2nd day)*
E.P.	23	F	Carcinoma colon	Triamcindone, 32 mg. daily for 3 months	0	0	
F.S.	62	F	Behcet's disease	Prednisone, 30 mg. daily for 5 weeks	0	0	
W.B.	55	F	Rheumatoid arthritis	Cortisone, 75 mg. daily for 3 years	6	9	
E.P.	60	F	Treated in error as Addison's disease	Cortisone, 37·5 mg. daily for 3 years	11	17	31
E.W.	44	F	Rheumatoid arthritis	Cortisone, 12·5 mg. daily for 3 weeks (previously 75-100 mg. daily for 7 years)	5	26	39

and relatively easy to perform, so that a dozen estimations can be carried out in a period of three hours, excluding the time required for the colour development. The convenience and simplicity of this corticotrophin zinc test is such that patients in whom the diagnosis of adrenal insufficiency is clinically suggestive can receive a preliminary assessment of their adrenal cortical capacity even on an out-patient basis.

This work was carried out while I was in receipt of a research grant from the Governors of St. George's Hospital.

I wish to thank Professor A. C. Dornhorst for helpful criticism, and the physicians of the hospital for permission to investigate their patients.

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The influence of penicillin on *Lactobacillus leichmannii* serum B_{12} assay

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SYNOPSIS The influence of penicillin on vitamin B_{12} assay using *L. leichmannii* as the test organism was investigated, and it was found that penicillin even in a low serum concentration invalidated the test.

The assay of vitamin B_{12} in serum is now accepted as a routine laboratory test. In some laboratories the method using *Lactobacillus leichmannii* as the test organism is used in preference to the more time-consuming *Euglena gracilis* method. It gives satisfactory results and does not require special equipment but the fact that *L. leichmannii* used in the test is sensitive to antibiotics is sometimes ignored and specimens for the test are collected during antibiotic treatment. The purpose of this investigation is to draw attention to possible errors in B_{12} assay by using the *L. leichmannii* method on sera collected while patients were receiving penicillin.

METHODS

B_{12} ASSAY IN SERUM A modification of methods described by Hawkins and Meynell (1958) and Cooper (1959) was used.

Medium Double strength Difco B_{12} assay broth was prepared according to the makers' instructions.

Preparation of serum extracts To 1 vol. of serum one fifth volume of 0·1% sodium cyanide and 1 vol. of acetate buffer, *p*H 4·6, were added. The mixture was left at room temperature for about 30 minutes and then distilled water was added to make a total of 10 vol. (If the water is added immediately after the buffer some extracts may be opalescent or milky; all extracts are water clear when the proper interval is observed.) The mixtures were placed in a boiling water bath for 30 minutes, cooled, and centrifuged. The clear supernatant extracts were used for the assay.

Preparation of inoculum A fresh, 24-36-hour-old culture of *L. leichmannii* in Difco inoculum broth was centrifuged and the deposit washed twice with sterile saline and then re-suspended in saline. The suspension was further diluted with saline till it was just slightly opalescent.

Procedure In the standard test two extract strengths are used, neat and diluted 1:2·5. In this investigation extracts were used, neat and diluted 1:2, 1:5, 1:10, 1:20, 1:50. Sterile vitamin B_{12} aqueous solutions containing 0 to 50 $\mu\mu$g. per ml. were prepared for the standard B_{12} curve. To 5 ml. aliquots of neat and diluted extracts and of B_{12} standard solutions in sterile tubes an equal amount of double-strength Difco B_{12} assay broth and 0·1 ml. of *L. leichmannii* inoculum were added. The tubes were incubated anaerobically at 37°C. for 36 hours. After incubation the cultures were diluted with an equal volume of 3% HCl in saline and the opacity measured in a Unicam SP 500 spectrophotometer (wavelength 590 millimicrons, large tube with light path of 24 mm.). Dilution with acid saline has two advantages: it confines all opacity readings in the test to the more sensitive part of the logarithmic scale and it produces a uniform yellow colour in all culture tubes, which, after incubation, are of different shades from reddish-brown to yellow.

DETERMINATION OF L. LEICHMANNII SENSITIVITY TO PENICILLIN A series of twofold dilutions of penicillin in sterile distilled water was prepared. The solutions contained between 0·0038 and 0·48 units of penicillin per ml. To 5 ml. aliquots of each solution an equal amount of double-strength Difco B_{12} assay broth containing 50 $\mu\mu$g of B_{12} per ml. and 0·1 ml. of *L. leichmannii* inoculum were added. The tubes were incubated anaerobically at 37°C. for 36 hours and the presence or absence of growth was read macroscopically.

PENICILLIN ASSAY IN SERUM AND SERUM EXTRACTS The method used was that described by Mackie and McCartney (1953). From both neat serum (or extract) and from its 1:100 dilution in sterile broth a series of twofold dilutions in sterile broth was prepared (from 1:1 to 1:64). At the same time a series of twofold penicillin dilutions in sterile broth containing between 0·0019 and 0·24 units per ml. was also prepared. Then 0·3 ml. aliquots of all serum (or extract) and of penicillin dilutions were inoculated

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with a standard loopful of 1:300 dilution in broth of a 24-hour-old broth culture of the Oxford staphylococcus. The tubes were incubated overnight and the presence or absence of growth observed macroscopically.

The lowest penicillin concentration that inhibited growth of the Oxford staphylococcus was invariably 0·03 units per ml. The same penicillin concentration was assumed to be present in the highest serum or extract dilution that inhibited growth of the Oxford staphylococcus.

RESULTS

The lowest level of penicillin in the serum to be assayed which might, by inhibiting the growth of the test organism, interfere with the assay depends on (1) the sensitivity of *L. leichmannii* to penicillin, (2) the dilution of the serum to be assayed, and (3) the loss of penicillin activity during preparation of extract.

L. LEICHMANNII SENSITIVITY TO PENICILLIN *L. leichmannii* was found to be resistant to 0·015 units per ml. and sensitive to 0·03 units per ml. penicillin. These limits are the same as for the Oxford staphylococcus and this fact was found useful in this investigation.

LOSS OF PENICILLIN ACTIVITY IN SERUM EXTRACTS To evaluate the deterioration of penicillin activity during the preparation and storage of extracts penicillin levels in 12 sera and their extracts were determined and compared. The results are given in Table I.

TABLE I

PENICILLIN CONCENTRATIONS IN SERA AND THEIR EXTRACTS

Sample	*Penicillin Content in 1 ml. Serum (units)*	*Penicillin Content in 10 ml. Extract (units)*	*Time between Collection of Serum and Preparation of Extract*	*Residual Penicillin Activity in Extract (%)*
1	4·8	2·4	Prepared from fresh serum	50
2	19·2	9·6	Prepared from fresh serum	50
3	3·0	1·2	Prepared from fresh serum	40
4	3·0	2·4	Prepared from fresh serum	80
5	3·0	1·2	5 days	40
6	24·0	9·6	5 days	40
7	6·0	4·8	5 days	80
8	6·0	2·4	5 days	40
9	24·0	9·6	10 days	40
10	2·0	1·2	10 days	60
11	2·0	0·6	10 days	30
12	48·0	19·2	10 days	40

Serum penicillin concentrations were estimated on fresh sera and again after they had been stored frozen at −40°C. for 10 days. There was no deterioration in penicillin activity after storage. On the other hand, some penicillin activity was lost during preparation of extracts. The residual activity was between 30% and 80% of that originally present in the serum. The double dilution method used for penicillin assay is not very accurate and, therefore, the figures in Table I give only approximate values.

L. LEICHMANNII ASSAYS ON SERA FROM PATIENTS RECEIVING PENICILLIN The inhibitory action of penicillin on *L. leichmannii* explains findings in routine B_{12} assay work when on some occasions the presence of penicillin in tested sera invalidated the test. The results obtained in five sera submitted for routine B_{12} assay from patients who were being treated with penicillin are shown in Fig. 1. A striking finding in these five tests was the complete inhibition of growth of *L. leichmannii* in tubes containing large concentrations of serum extracts. These are the concentrations used in routine B_{12} assay

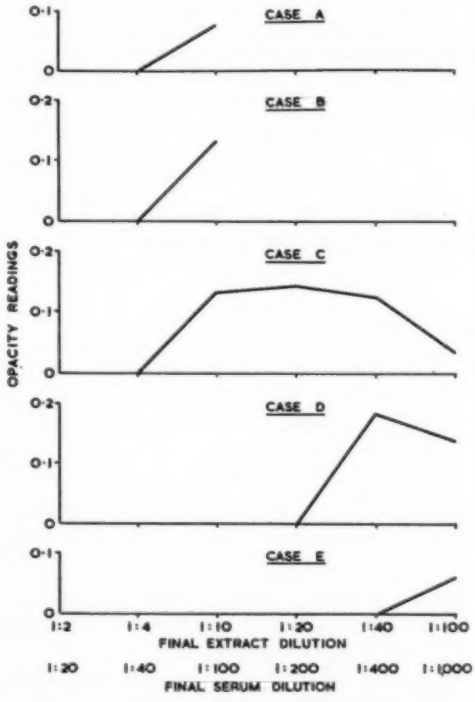

FIG. 1. *Opacity readings in B_{12} assay on five sera collected during penicillin treatment. Penicillin content in extracts: Case A, 0·06 units/ml.; Case B, 0·12 units/ml.; Case C, 0·24 units/ml.; Case D, 1·0 unit/ml.; Case E, 1·9 unit/ml. Penicillin levels in sera: Case A, 2·0 unit/ml.; Case E, 48·0 unit/ml.; there was insufficient serum in the remaining three cases.*

(extracts diluted 1:2 and 1:5 corresponding to serum dilutions of 1:20 and 1:50). The inhibition corresponded roughly to the pencillin level in the extract. In Cases A, B, and C, in which extract penicillin levels were less than 0·25 units per ml., the growth of *L. leichmannii* was completely inhibited in the first two tubes only. In Case D, in which extract penicillin level was 1 unit per ml., there was no growth in the first four tubes, and in Case E with an extract penicillin level of 1·9 units per ml. in the first five tubes. Such complete lack of growth in culture tubes inoculated with *L. leichmannii* is never found when sera are free from penicillin. Even with sera from patients with pernicious anaemia in those tubes containing the least amount of extract there is always a slight turbidity visible to the naked eye.

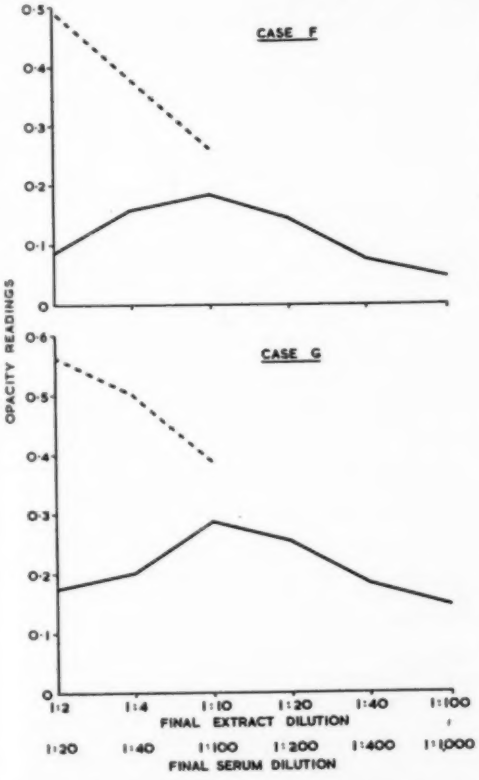

FIG. 2. *Opacity readings in serum B_{12} assays before and during penicillin treatment (interrupted line — — — before, continuous line———during penicillin treatment). Penicillin doses, serum penicillin levels, and calculated serum B_{12} values are given in Table II.*

While penicillin interference should not be missed in cases where growth of *L. leichmannii* is completely inhibited, it is less easily recognized in tests where extract penicillin levels are so low that growth of *L. leichmannii* is only partly inhibited. Two such cases are shown in Fig. 2. These two patients were treated with fairly low doses of penicillin (Case F, Broxil tablets, 500 mg. daily, and Case G, penicillin injections, 500,000 units daily). Two blood specimens for B_{12} assay were collected from each patient, one before and another on the second day of penicillin treatment. It was assumed that there would be no significant difference in B_{12} levels between two specimens collected at such short intervals. There was a marked difference in the appearance of opacity curves between the first and the second specimens. The specimens collected before penicillin treatment showed a more or less regular slope, while the specimens collected during treatment produced a curve of unusual shape. The growth of *L. leichmannii* was scanty in the first tubes containing undiluted extract. It increased with each dilution till the peak was reached and then diminished with further dilutions. The first, rising part of the curve was obviously related to penicillin and not to B_{12} concentration. The peak values in both cases were less than in corresponding dilutions of penicillin-free extracts and could not be accepted as representing true B_{12} concentrations. The serum B_{12} levels calculated from each dilution are shown in Table II.

TABLE II

SERUM B_{12} VALUES CALCULATED FROM OPACITY READINGS SHOWN IN FIG. 2

Case	*Sample*	*Penicillin Dose*	*Serum Penicillin Level (units/ml.)*	*Extract Dilutions*[1] ½ (1/20)	¼ (1/40)	⅛ (1/100)
F	1	—	—	510	480	500
	2	Oral Broxil, 500 mg. daily	0·06	10	80	250
G	1	—	—	[2]	1000	1100
	2	Penicillin, 500 u. i.m. daily	0·12	32	700	550

[1]Corresponding serum dilutions are shown in brackets.
[2]Opacity too high, outside standard curve range.

Dilutions in the descending part of the curve were outside the useful range. It was also possible that the growth of *L. leichmannii* in these tubes was still influenced by traces of penicillin.

DISCUSSION

The examples described above show that the low concentrations of penicillin in serum, which can be

expected in practically every case treated with penicillin, will invalidate B_{12} assay by the *L. leichmannii* method. When the penicillin level is high enough to cause complete inhibition of growth the fact is easily noticed. A partial inhibition of growth by smaller concentrations of penicillin in serum may not attract attention and consequently false low values may be erroneously reported.

When the serum B_{12} level has to be estimated during penicillin treatment it should be done by the *Euglena gracilis* method. Ross (1952) found that 'sulphathiazole, penicillin, streptomycin, aureomycin, chloramphenicol, and para-aminosalicylic acid when added in concentration likely to be found in human serum during treatment with these drugs were without significant effect on growth of *Euglena gracilis*'. Lear, Harris, Castle, and Fleming (1954), on the other hand, reported that 'sulphonamide derivatives may be present in serum in concentrations which can inhibit *Euglena* growth'. Hutner, Bach, and Ross (1956) showed that the addition of para-aminobenzoic acid to the basal medium abolished the inhibitory effect of sulphonamides but did not interfere with the growth of *Euglena gracilis* in serum B_{12} assay.

I wish to thank Dr. C. L. Greenbury for his encouragement and advice in the preparation of this paper.

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Human listerial meningitis

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SYNOPSIS A case of Listerial meningitis is described in a man of 60 years. The aetiology of listeriosis is discussed.

Human listeriosis is widespread throughout the world (W.H.O. Report, 1959), some 400 cases having been reported so far (Seeliger, 1958), but its existence has been neglected in Great Britain. Only some eight cases have been recorded (Lane, Watling, and Marshall, 1959), the majority in infants. Seeliger thinks that there has not been any real increased incidence but considers that there has been a greater awareness of the condition recently.

CASE REPORT

F.E.L., a married man, aged 60 years, worked as a plant attendant at the Northern Sewage Outfall Works, Barking. He was admitted on 15 September 1959 with severe headache and vomiting for three days. He had had no major illnesses.

On examination, his temperature was 100°F., pulse 88, respiration 18. He was irritable and delirious. Neck stiffness and Kernig's sign were present. Blood pressure was 130/70 mm. Hg. An aortic systolic murmur was heard. The glans penis was red and swollen and discharging from the preputial space. There were no other signs.

INVESTIGATIONS On admission the cerebrospinal fluid was turbid containing 800 cells per c.mm. (80% polymorphonuclears), protein 200 mg. %, sugar 20 mg. %, and chloride 660 mg. %.

When the examination was repeated on 26 August, the cerebrospinal fluid was slightly turbid, containing 225 cells per c.mm. (90% lymphocytes), protein 60 mg. %, and chlorides 660 mg. %. No growth was obtained on culture.

Blood culture showed Gram-positive bacilli identified as *Listeria monocytogenes* type 4B. Haemoglobin was 102% (14·9 g. %), white cells 12,700 per c.mm. (84% polymorphonuclears).

A swab taken from the preputial space showed no growth and urine was sterile on culture.

An E.C.G. was normal.

Paper strip electrophoresis gave total protein, 8·5 g. % (albumin 4·75 g. %, α_1 globulin 0·35 g. %, α_2 globulin 0·85 g. %, β globulin 1·05 g. %, γ globulin 1·5 g. %). The pattern was abnormal and the α_2 and β globulin fractions abnormal.

Detailed bacterial studies were performed on blood and cerebrospinal fluid and both yielded a growth of small (1 mm. overnight) greyish low-convex colonies, with an entire edge, surrounded by a zone of slight β haemolysis. Morphologically this was a Gram-positive bacillus (approximately 2·5 × 0·5 μ) with rounded ends. Some of the organisms presented in pairs and a few in long chains. The organism grew aerobically at 37°C. and at room temperature. The 37°C. cultures in liquid media showed no motility, but a culture in peptone-water incubated overnight at room temperature was sluggishly motile, and, in peptone-water/maltose it showed a moderately active 'tumbling' motility. On solid media the organism behaved as follows:

Media	*Room Temperature*	*37°C.*
Nutrient agar, 18 hr.	Slight growth	Moderate growth
Blood-agar, 18 hr.	Scanty growth with slight zone of β haemolysis	Moderate growth with distinct zone of β haemolysis
MacConkey's agar, 18 hr.	No visible growth	Growth just visible
MacConkey's agar, 72 hr.	Visible growth	Visible growth
Gelatin stab, 18 hr.	Growth along line of stab	—
Gelatin stab, 1 week	No liquefaction	—
Loeffler's serum, 18 hr.	Growth	Growth
Loeffler's serum, 1 week	No liquefaction	No liquefaction
Urease production, 1 week	—	—
Indole production, 1 week	—	—

Serum-broth cultures incubated for eight or 10 hours at 37°C. produced a soluble haemolysin active against horse red cells. Its fermentative characteristics at both 37°C. and room temperature were:

	Glucose	*Lactose*	*Dulcite*	*Sucrose*	*Mannite*	*Maltose*
18 hr.	—	—	—	—	—	—
36 hr.	Acid, no gas	—	—	—	—	—
48 hr.	Acid, no gas	Acid, no gas	—	—	—	—
1 week	Acid, no gas	Acid, no gas	—	Acid, no gas	—	—

SENSITIVITY TO ANTIMICROBIAL AGENTS *In vitro*, the organism was highly sensitive to sulphafurazole, penicillin, streptomycin, erythromycin, chloramphenicol, tetracycline, neomycin, and oleandomycin. The organism

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was presumed to be *Listeria monocytogenes* and the N.C.T.C. 7974 strain was obtained for comparative agglutination tests, which were placed in the 37°C. water-bath for 18 hours and then stored overnight at about 10°C. before final readings were made. Formolized and alcoholized suspensions of the patient's organism and of the N.C.T.C. strain gave the following titres (expressed as reciprocals) in H and O agglutination tests respectively.

	Patient's Organism	*N.C.T.C. 7974*
Patient's serum and H suspension	160	40
Patient's serum and O suspension	80	80
Normal serum and H suspension	10	20
Normal serum and O suspension	Less than 10	10

The strain was tested by Dr. Edmunds (Edmunds, Nicholson, and Douglas, 1957) against absorbed factor specific *L. monocytogenes* sera. There were positive reactions with H factor A (25°C. cultures giving good H agglutination) and against O factor V, but not against H factor D and O factors I and IV.

TREATMENT On admission, benzyl-penicillin, 1 mega unit six hourly, was given and continued for two weeks, totalling 56,000,000 units, and, sulphadimidine, 4 g. followed by 2 g. six hourly for 24 hours; on the following day chloramphenicol, 2 g. daily, and streptomycin sulphate were started and 2 g. given daily for nine days, a total of each of 19 g.; then intravenous dextrose (4·82%) with saline (0·18%), each 500 ml. containing 100 mg. hydrocortisone, were given to a total of 2,400 ml.; and oral prednisone, 10 mg. six hourly, gradually tailing off to a total of 140 mg.

PROGRESS During the first 24 hours he had to be catheterized twice but the temperature became normal within this time. Within three days he was well and was discharged symptomless on 12 September. Three months later he was still well and back at work not as a sewage worker but in the power house.

DISCUSSION

A purulent meningitis of uncertain origin was first diagnosed. As in other cases, the organism was sensitive *in vitro* to many agents. In view of the number of drugs used, no conclusion as to their individual efficacy was possible. Seeliger, however, considers tetracycline to be the drug of choice. Steroids were given early because of their reputed anti-toxaemic action and because some petechiae were seen which suggested early adrenal insufficiency.

The source of infection remains obscure in human beings and in animals. In both, isolated single cases are not uncommon. Though the morbidity is low, mortality in affected animals is high. Like human beings, young animals are more susceptible than adults, the disease being more rapid in them. In human beings, however, the outlook is said to be worse in those over 60 years of age.

In poultry a lowering of flock resistance, *e.g.*, by parasites or salmonellosis, seems necessary before *Listeria* can cause high losses. In cattle the disease is commoner when fresh green food is scarce. The difficulty of reproducing the disease artificially suggests that unknown factors are present. Brennaas and Brochmann (1959) report a fatal case in a man with lymphosarcoma. Jessen (1958) thinks the association with malignant disease is common in human listeriosis. There are several reports of affected animals being eaten without ill effects by stockmen and farmers. Ödegaard, Grelland, and Henriksen (1952) suggest that a farmer acquired a fatal infection from healthy ovine carriers, but infection might equally well have been from farmer to sheep.

The positive blood culture in this case suggests entry through the skin, possibly of the hands or feet when the patient took off his boots; a number of cases have been reported in shoe repairers. Bigland (1950) isolated the organism from canaries and chickens. Our patient kept two budgerigars but examination of their droppings was negative.

Septicaemia with enlarged lymph glands occasionally occurs in this disease and may be confused with infectious mononucleosis (Girard and Murray, 1951). In the present instance there was a polymorph leucocytosis. Many authors hold that the incubation period is exceptionally long for bacterial disease, but this is surely difficult to substantiate in the absence of any known source of infection. Others describe a chronic course of weeks or months, unlike the one month's duration of the present case (Seeliger and Cherry, 1957). Meningeal involvement is the common presenting form in adults, and it seems likely that it is secondary to a blood stream infection as in this patient.

Listeria has been recovered from the urethra of some adult males, but despite the presence of active inflammation, no organisms were isolated from this patient's urine or prepuce. Wedemeyer and Seeliger (1959) found an increased serum antibody titre in the mothers of five neonatal cases. A titre of 1 in 320 was regarded as significant. The relevant titres in this case were O 1 in 80 and H 1 in 160, but it should be remembered that the presence of a raised titre alone is insufficient for diagnosis. Wedemeyer and Seeliger (1959) also stress that the maternal titre is not increased in all proven neonatal infections, overt disease being rare in mothers; this seems to weaken the theory of venereal spread sometimes put forward.

All reports agree that in neonates liver necrosis is a prominent feature unlike the picture in adult meningitis. Meconium is said to swarm with organisms but Wedemeyer and Seeliger (1959) dis-

agree. They stress pre-culture of meconium at 4°C. for six weeks (Gray's method), a positive culture having been obtained in one of their cases after five weeks. These authors also state that the organism has been isolated from the placenta, cervix, and vagina both in human beings and animals. Winn, Cherry, and King (1958) stress that questioning the mother after abortion or premature birth frequently reveals some preceding influenzal-like symptoms.

The occurrence of conjunctivitis in laboratory workers may be of some importance. Although the source of infection has been undetermined, rubbing the eyes with an infected hand seems an obvious route.

Serum electrophoresis was abnormal in the present case, the α_2 and β globulin fractions being increased. Whether these fractions carry antibody or are related to the increased titres described previously is conjectural. According to Edmunds (personal communication), serological typing is not of much value epidemiologically. Most of the recorded cases in this country are type 2 or 4b as in this instance, but differentiation of the five subtypes of type 4 is difficult.

In conclusion, Jessen (1958) optimistically thinks that the mortality from this disease, especially meningitis, has been significantly reduced by antibiotics, but this surely remains to be seen.

It is a pleasure to thank Mr. L. Whittaker for his perspicacity in isolating the organism, and Dr. T. F. Elias-Jones (group bacteriologist) for performing the extensive range of confirmatory tests and for permission to use the laboratory findings. Thanks are also due to Dr. P. N. Edmunds, formerly of Edinburgh, for serotyping the organism, and to Dr. R. W. Tannahill for his permission to publish this case and for his encouragement. We are also grateful to Dr. W. B. Cherry, of the Public Health Service, Georgia, U.S.A. for his help with the references.

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Death of an infant in hyperthermia after vaccination

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SYNOPSIS Death following hyperpyrexia and coma of rapid onset nine days after vaccination of an infant is reported. Death appeared to be due to overwhelming vaccinial infection without encephalitis. Large amounts of virus was present in lung, spleen, bone marrow, and the vaccination site.

Three sometimes fatal complications of vaccinia are well known: post-vaccinial encephalitis, generalized vaccinia, and progressive vaccinia. This report describes a fourth variety.

CLINICAL HISTORY

A baby girl, 3 months old, was vaccinated on the upper arm by a general practitioner using the 'multiple pressure' method. The baby was seen by a health visitor on the seventh day after vaccination and appeared well; the vaccination showed a normal 'take'. The parents reported that on the evening of the eighth day she appeared well; at 6.30 a.m. the following morning she 'looked all right'; at 8.30 a.m. the child could be roused, but was very sleepy; at 9 a.m. she could not be wakened, and when the doctor arrived at 9.10 a.m. the child was dead. The body felt extremely hot to the touch and was steaming. The rectal temperature was at least 108°F. (42·2°C.), the highest figure on the thermometer scale.

NECROPSY FINDINGS

Externally the body was that of a well-nourished, well-cared for female infant, with the blister of a recent vaccination on the left upper arm. The tongue was clenched tightly between toothless gums. Both hands were clenched and the arms rigidly flexed. Although death was stated to have occurred seven hours previously, the temperature of the body was 97°F. when it was opened in the unheated post-mortem room in late November.

Within the body, there were very extensive asphyxial haemorrhages in the pericardium, thymus, and pleura. The heart and lungs were otherwise normal except that the airways were filled with a very fine white froth. There was congestion and cloudy swelling of liver and kidneys, and a congested spleen. There was no evidence of any sepsis beneath the vaccination wound, and no evident abnormality in the alimentary tract.

The brain showed extreme pink suffusion of the grey and white matter.

LABORATORY INVESTIGATIONS

Necropsy specimens were taken from the following organs for histology and for virological examination: Cerebral cortex, kidney, spleen, a rib (for bone marrow), lung, heart, and the skin bearing the vaccination lesion. For histology the tissue was fixed in formal saline and for virology was stored at −30°C. until eggs and tissue cultures were ready for inoculation. The skin lesion was all taken for virus titration and was not examined histologically.

Suspensions of tissue, 10-20% (w/v), were made by grinding with sterile pyrex powder in a mortar and were titrated in eggs using eight eggs per 10-fold dilution by the method of Overman and Tamm (1956); as a check, infectivity titres in terms of TCD_{50} for ERK 1 cells (Westwood, Macpherson, and Titmuss, 1957) were also determined, using four tubes per 10-fold dilution. The infectivity end-point was determined as shown after seven days' incubation, though it is possible that with further incubation the titre might have appeared higher. The titres are shown in Table I. No virus was isolated from brain or kidneys either in eggs or tissue culture. There was no evidence of virus other than vaccinia in any specimen,

TABLE I

TITRES OF TISSUES EXAMINED

	Skin from Vaccination Site	*Spleen*	*Lung and Heart Pooled*	*Rib*
Pock-forming units/g. tissue	$8·7 \times 10^6$	$3·1 \times 10^5$	$3·2 \times 10^4$	$4·7 \times 10^5$
TCD_{50}/g. tissue	10^6	10^5	10^5	10^5

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No sample of blood was taken during the course of the illness and neutralizing antibodies were not looked for.

HISTOLOGY

Unfortunately no sections of the lung were available. The only changes visible in the other organs were acute cloudy swelling of liver, kidney, and brain; the blood vessels in the brain appeared very congested. No inclusion bodies were found. (The skin lesion was not examined.) There was no evidence of perivascular fragmentation or loss of myelin or of acute inflammatory change in the brain.

DISCUSSION

The recognized complications of vaccination are post-vaccinial encephalitis, generalized vaccinia (usually associated with eczema), and progressive vaccinia (a rare disease, possibly associated with agammaglobulinaemia).

The course of events in this case differs from any of these. The interval between vaccination and death was shorter than that described in well-authenticated reports of post-vaccinial encephalitis. It seems most probable that there was an acute overwhelming vaccinial infection with associated toxaemia leading to hyperpyrexia and death. This, however, does not entirely explain the asphyxia, which we can only account for by postulating spasm of the bronchioles, perhaps as an allergic reaction to large quantities of vaccinial antigen. The reaction appears to be an individual one; another child inoculated on the same day by the same practitioner with the same batch of vaccine behaved normally. As far as we are aware, no report of a precisely similar fatality due to vaccination has been published. It may be that they have been diagnosed as 'vaccinial encephalitis'. Such a provisional diagnosis was made in this case until it became clear, on histological examination of the brain, that there was no encephalitis. Dr. Marmion has, however, told us of a somewhat similar death with hyperpyrexia (106°F. (41·1°C.) recorded) in an infant nine days after vaccination, which he investigated serologically. In this case, though intensive attempts were made to isolate vaccinia, enteroviruses, and influenza virus, all cultures were negative except those from the vaccination site from which vaccinia virus was isolated. At necropsy, there was no evidence of encephalitis but some evidence of pneumonia with a mononuclear cell exudate.

The lesions on the chorioallantoic membrane produced by the virus isolated from this patient were indistinguishable from those produced by vaccine lymph. They did not show any unusually marked necrosis in the centre of the pocks, as has been described for 'neurovaccinia'.

The investigation of this case has been by no means as complete as we could have wished. We are reporting it in the hope that pathologists will be on the look-out for such cases and will undertake a more detailed virological and histological examination of any that may occur.

Our thanks are due to Dr. B. P. Marmion, Dr. Denis Harriman and Dr. Kenneth Froome for information about patients investigated by them, and Professor A. W. Downie, F.R.S. for his comments.

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Technical methods

Determination of packed cell volume by centrifugation

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In a recent note Hutchison (1960) has commented on the determination of the packed cell volume and has drawn attention to the importance of the relative centrifugal force as a measure of centrifugal efficiency. Since the relative centrifugal force in *g* for unit mass is given by the expression,

$$F = mw^2r$$

where w is the angular velocity and r the radius of gyration, it is clear that since the radius occurs only as a first order quantity and the angular velocity as its square, the latter plays a more important part. This is undoubtedly the reason why 'the figure of 3,000 r.p.m. for 30 min. is too often all that is thought important' rather than 'the force applied by the centrifuge in *g*' (Hutchison, 1960).

If centrifugation is continued for a sufficiently long time at any speed, the volume of packed cells will reach a constant after a certain time and prolonged spinning after this time will not reduce this volume. The time required will vary from a few minutes to several hours dependant on the relative centrifugal force employed. This does not mean, however, that the cells will be completely packed (Ponder, 1948a) and to regard attainment of constant volume as a criterion of complete packing is quite erroneous.

Millar (1925) used an experimental haematocrit centrifuge in which the rotational speed, and hence the relative centrifugal force, could be varied up to a maximum of approximately 30,000 *g* and spun samples of oxalated blood at each of several speeds until constant volume was attained. On plotting a graph of cell volume per cent against relative centrifugal force he found that the curve approached an asymptotic line parallel to the relative centrifugal force axis which represented the actual cell volume that would have been attained if the centrifugal force had been indefinitely increased. His results show that at 3,000 r.p.m. with a radius of 10 cm. (relative centrifugal force 1,370 *g*) the cells are packed to within approximately 1% of their final value. The criticism that very high centrifugal force might cause rupture and also would tend to compress the cells to give a low value for the packed cell volume can be rejected (Ponder, 1948b). Indeed, Parpart and Ballentine (1943) have used centrifugal forces of up to 200,000 *g* and have found that, even after packing under these great forces, the cells are unharmed and can be resuspended and centrifuged many times to give the same result. From these results it would appear likely that true values for packed cell volume, that is, complete packing so ensuring exclusion of all plasma from cell interstices, can only be achieved by prolonged spinning with relative centrifugal forces of several hundred thousand times gravity.

That this is impracticable in the clinical laboratory is obvious and is indeed unnecessary. Ponder uses the original Wintrobe (1942) method in his clinical work which involves spinning at 3,000 r.p.m. for 15 min. originally in a centrifuge with a radius of 15 cm. (relative centrifugal force, approximately 1,500 *g*). To quote Ponder (1948b): 'It must be borne in mind that the red cell volume in men may range from 82 μ^3 to 95 μ^3 without its being considered abnormal; small errors in the estimation of volume are accordingly not very important'.

It is interesting to note that Wintrobe (1951) changed his technique in the third edition of his book to spinning at 3,000 r.p.m. for 30 min. but still used a small centrifuge. In the latest edition (1956a) he retains the 3,000 r.p.m. and 30 min., but has now changed his centrifuge to one of greater radius (22·5 cm.) and hence has a higher relative centrifugal force (2,260 *g*). The normal values of packed cell volume given by Wintrobe remain the same.

The position is further confused by descriptions in various standard textbooks of methods purporting to be Wintrobe's technique but all differing in some detail.

The range of normal values given also shows wide variations. Thus Wintrobe gives a normal range in males of 47% ± 7% and technique as above; May and Marrack (1951) a normal range of 42% to 47%, technique 3,000 r.p.m. for 30 min., no radius stated; Wells (1956) spins at 2,500 r.p.m. to 3,500 r.p.m. for 30 min. then for a further 10 min. to ensure complete packing; Whitby and Britton (1957) spin at 3,000 r.p.m. but for 55 min.; Darmady and Davenport (1958) spin at 3,000 r.p.m. for 30 min. but give a normal range of 40% to 50% for males; Delaney (1960) is specific with 3,000 r.p.m. for 60 min. at a radius of 15 cm., normal values being 40% to 50%, and Soiverd (1958) spins at 3,000 r.p.m. for 30 min. or 'at a high speed until two successive readings are identical'. A number of other variants to the test, all regarded as satisfactory by the respective observers, can be found in the literature, and at least 10 of these are quoted by Ponder in his book.

Despite these anomalies it is surprising to find that Hutchison considers that the fluctuations in M.C.H.C. of a patient could be attributed to inaccuracies in packed cell volume estimations rather than in Hb estimations. Although it cannot be denied that the Wintrobe technique does not give absolute values of packed cell volume, all authors agree that it is reproducible. Indeed any centrifugal technique in which a standard time and standard relative centrifugal force are employed must be, since such variable parameters as viscosity, temperature, and

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so on, are, in this context, negligible. Many haemoglobin methods are by no means so reproducible, the dilution methods being particularly unreliable. An authoritative study of these methods made for the Medical Research Council (Macfarlane, King, Wootton, and Gilchrist, 1948) found that even with a single observer no significance could be attached to differences of less than 10%. In addition haemoglobin pipettes purchased on the open market may carry an error as great as 5% (Lochhead and Purcell, 1952). (An excellent review of errors in Hb estimation can be found in Wintrobe's book, 1956a.)

With regard to the question of errors in reading haematocrit tubes, it should be noted that Wintrobe (1956b) considers this to be so insignificant that he suggests that certain small angle centrifuges may be used for packed cell volume determination 'which have the small disadvantage that the cells in the haematocrit are packed at an angle and the true value must therefore be estimated'.

A source of potential error in the determination of packed cell volume is that of sampling. Blood samples, particularly those with a high E.S.R. may sediment very rapidly and appreciable differences in cell content between top and bottom layers can be detected in some cases after only a few minutes.

Finally the question of the M.S.E. haematocrit centrifuge: this machine was designed some 10 years ago to conform with Wintrobe's specification at that time. The radius of gyration is 137 mm., and a governor may be switched into circuit so that the required speed of 3,000 r.p.m. may be accurately maintained. An automatic time switch is incorporated to facilitate accurate timing. Under these conditions this centrifuge will provide reproducible packed cell volumes which conform to what is generally accepted as Wintrobe's test. Nevertheless, should the mains voltage be above 230 v then the machine can be operated at 3,800 r.p.m. with the governor switched out of circuit. This will provide a relative centrifugal force of 2,264 *g* as required by the latest modification of Wintrobe's test. If required a simple modification can be made so that a governed speed of 3,800 r.p.m. can be maintained. Should the mains voltage be below 230 v, a simple voltage transformer will be required. However, practical tests show that this modification cannot be regarded as worthwhile. It is evident that the greater the true volume of packed cells, the greater will be the difference in the final reading between the two methods. Comparing tests at 2,264 *g* and 1,372 *g* respectively with a specimen of high packed cell volume, the results were 49% and 50%. The haemoglobin content was 16·5 g./100 ml. and the M.C.H.C.s were therefore 33% and 33·7%. In clinical work this is obviously insignificant, especially when one considers that neither method can be regarded as absolute.

CONCLUSIONS

The determination of packed cell volume is a clinical test and absolute values require a relative centrifugal force, much greater, of up to 200,000 *g*. The clinical test is extremely reproducible and absolute values are of no significance in clinical work because of the wide range of normal values.

Errors in M.C.H.C. determination are more likely to be due to errors in the haemoglobin determinations than in estimates of packed cell volume.

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Tris buffer for differentiation between haemoglobins C and E and separation of haemoglobins S from F and 'lepore' from A

H. LEHMANN AND A. SHARIH *From the Department of Pathology, St. Bartholomew's Hospital, London*

Trishydroxymethylaminomethane (TRIS) buffer was introduced for the finer separation of serum proteins by paper electrophoresis by Aronsson and Grönwall (1957). Cradock-Watson, Fenton, and Lehmann (1959) found this buffer not suitable for the paper electrophoresis of haemoglobin variants but reported that with a slight, but important, modification it could be used to demonstrate haemoglobin A_2. We should now like to report three further uses for this modified buffer in the investigation of haemoglobins.

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DIFFERENTIATION BETWEEN HAEMOGLOBINS C AND E

On paper electrophoresis with barbiturate buffer *p*H 8·6 haemoglobin C and haemoglobin E have a very similar mobility. It is often difficult to differentiate between the two, although it can usually be established that haemoglobin C moves more slowly. The modified TRIS buffer can be used to help in distinguishing C and E in two ways.

(1) Whereas with barbiturate buffer, E (and A_2) are seen to move in front of C, the position is reversed with TRIS buffer (Figs. 1 and 2), where E and A_2 move more slowly than C. Thus the examination of an unknown haemoglobin mixture, together perhaps with a haemoglobin A+C control by paper electrophoresis with both buffers, will give a reliable identification.

(2) If haemoglobin S is added to a sample which contains C or E respectively, paper electrophoresis with barbiturate buffer will show a separation from S in both, but TRIS buffer paper electrophoresis will show S and C moving as a single band, and S and E as separate bands.

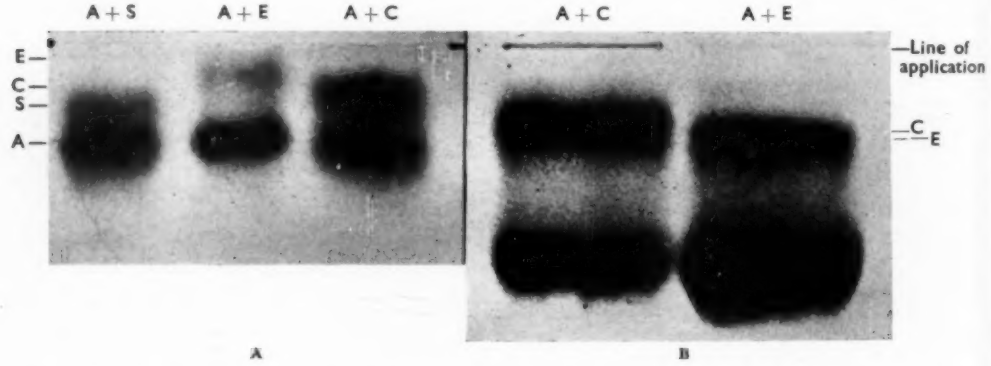

FIG. 1A. *Unstained strip showing from left to right haemoglobins A+S, A+E, and A+C. Paper electrophoresis using TRIS buffer. E moves more slowly than C.*

FIG 1B. *Stained strip showing from left to right haemoglobins A+C and A+E. Paper electrophoresis using barbiturate buffer. C moves more slowly than E.*

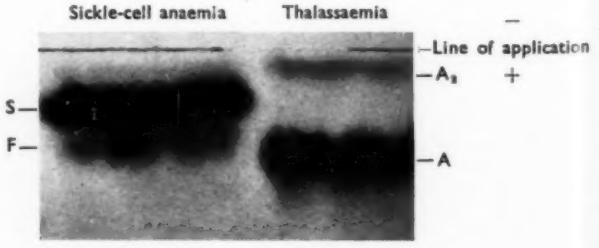

FIG. 2. *Unstained paper strip showing result of TRIS buffer electrophoresis. Left: Sickle-cell anaemia—S+10% F; note that A_2 can be recognized. Right: Thalassaemia minor—A, 1·2% F (not separating from A), 3·8% A_2.*

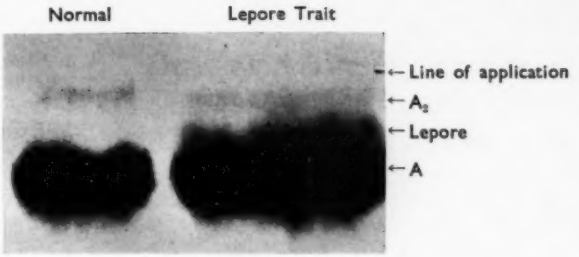

FIG. 3. *Stained (light green) paper strip showing the result of TRIS buffer electrophoresis. Left: normal—A and A_2. Right: Lepore trait—Lepore haemoglobin between A and A_2.*

In other words TRIS buffer paper electrophoresis fails to separate C and S, but can separate E and S.

SEPARATION OF HAEMOGLOBINS F AND S

The second advantage of TRIS buffer is that, unlike barbiturate buffer, it will separate haemoglobin F from haemoglobin S (Fig. 3).

SEPARATION OF HAEMOGLOBIN 'LEPORE' FROM A

One of the characteristics of Lepore is that it can be separated from A by starch electrophoresis with barbiturate buffer, but either not at all, or only very poorly, by paper electrophoresis (Gerald and Diamond, 1958). Paper electrophoresis with TRIS buffer separates the two haemoglobins.

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A simple spectrophotometric method for the determination of urea in blood and urine

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Several methods for determining urea in blood and urine are in use in clinical laboratories, but none is ideal in mass routine analysis. The spectrophotometric method based on a modified Ehrlich reagent proposed by Watt and Chrisp (1954) for pure solutions is therefore of interest because it can be modified for use in mass clinical analyses of blood and urine. Brown (1959) has modified the method for use on blood, but his procedure involves treatment with urease as well as spectrophotometric measurement and is hardly simple enough for routine clinical pathology laboratories. In this laboratory a modified Watt and Chrisp method has been used as a routine procedure for a year, and proved simple and reliable except in specimens from patients receiving sulphonamides or para-aminosalicylic acid, when it is unsatisfactory. Such specimens are easily recognized because of the atypical colour reaction (bright citrus colour) with the modified Ehrlich reagent. Other laboratories might therefore be interested in a detailed description of the technique.

REAGENTS

(1) SOMOGYI'S ZINC SULPHATE SOLUTION Crystalline zinc sulphate, 12·5 g., dissolved in 125 ml. 0·25 N sulphuric acid and diluted to 1,000 ml. with distilled water.

(2) 0·75 N SODIUM HYDROXIDE

(3) ALCOHOLIC EHRLICH REAGENT p-Dimethylaminobenzaldehyde (analytic), 20 g., dissolved into 1,000 ml. 96% alcohol. To this solution is added 100 ml. concentrated hydrochloric acid (analytic) and mixed.

(4) STANDARD SOLUTION Urea, 100 mg., is dissolved in distilled water, 100 ml., in a measuring flask.

PROCEDURE FOR BLOOD

Twenty-four millilitres of reagent (1) are run from a burette into a 100 ml. Erlenmeyer bottle. To this is added 3 ml. whole blood (stabilized by oxalate), the bottle is shaken to obtain complete haemolysis, and 3 ml. of reagent (2) added and the mixture shaken again. It is then filtered through a special filter (Schleicher and Schull No. 589³, Blauband) to remove the precipitated proteins. (It is important to use a suitable filter paper because the usual laboratory filters may give falsely increased values.) Of the clear and colourless filtrate 10 ml. is pipetted into a 25 ml. measuring flask and 10 ml. reagent (3) added. After mixing dilute with distilled water to the mark and renew mixing. After 10 minutes the mixture is ready for reading. The colour is stable, according to Watt and Chrisp, for 11 days.

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READING

The reactions are read in a spectrophotometer at 420 nm. with a blank prepared in the same way as the reaction mixture but with 3 ml. of distilled water instead of 3 ml. of blood. The urea standard (reagent 4) is treated in the same way as the blood. Cuvettes of 1 cm. thickness are suitable.

If p, s, and b are the extinctions of the sample, the standard, and the blank respectively, the urea concentration is

$$100(p-b)/(s-b) \text{ mg./100 ml.}$$

A Hilger Uvispec photoelectric spectrophotometer was used in this laboratory, as attempts to use a Bausch and Lomb Spectronic were unsuccessful. This is in good agreement with the finding of Brown (1959) that a Coleman Junior spectrophotometer was not suitable for reading the Watt and Chrisp reaction.

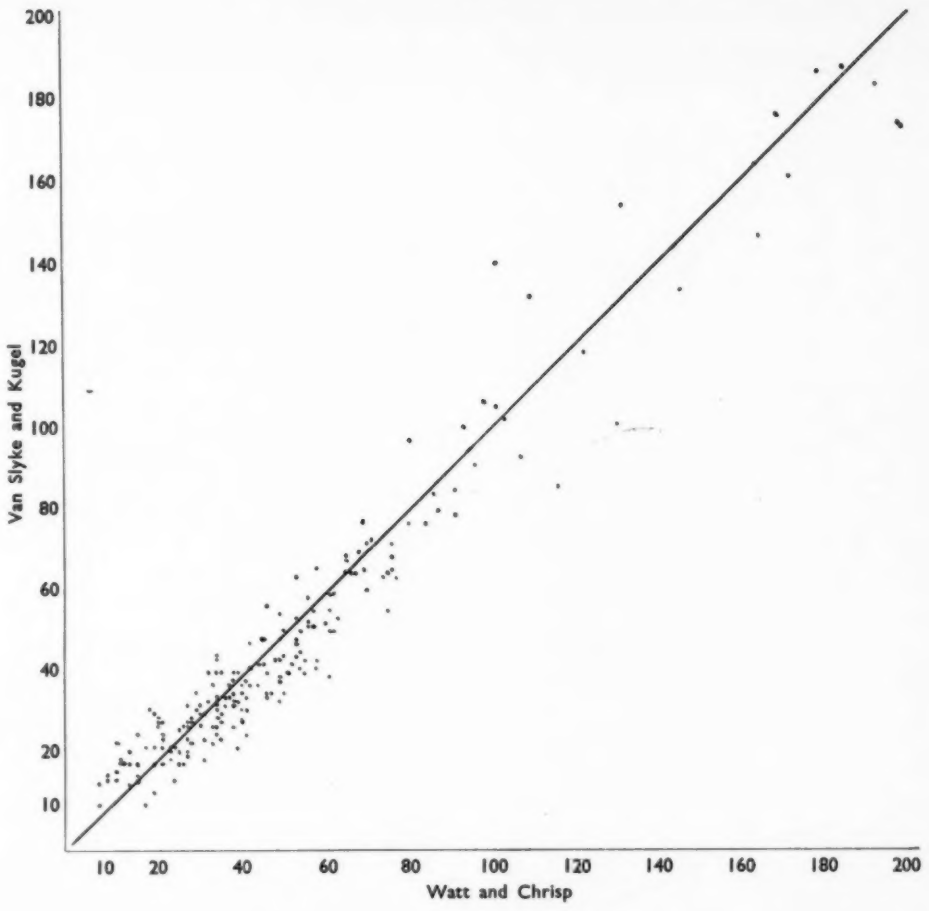
DETERMINATION IN URINE

The procedure is exactly the same as for blood, the only difference being that if the concentration is above 1,000 mg./100 ml. the reaction has to be repeated on a diluted sample because Beer's law is not valid for concentrations above this limit. The urine filtrate is quite colourless with the technique described.

COMMENT

The blank extinction is high compared with that of the reaction mixture and may vary somewhat from day to day for the same Ehrlich reagent. For different batches of Ehrlich reagent the blank extinction may vary between 0·070 and 0·170. According to our experience Ehrlich reagents giving a blank extinction below 0·100 are unsuitable. The high blank value is a drawback, but the method is, in spite of this, sufficiently accurate for clinical purposes.

To test the clinical reliability of the method 200 duplicate analyses were performed, one with the modified Watt and Chrisp method and one with the hypobromite method of Van Slyke and Kugel (1933). In the diagram the analyses with the Watt and Chrisp method are plotted along the abscissa, those with the Van Slyke and Kugel method along the ordinate. It is easily seen that the points are scattered evenly on both sides of the identity line. All the analyses shown in this diagram were routine clinical analyses. The diagram shows that the deviations between the two methods are too small to be of any clinical significance. Serum creatinine determinations have been made on all samples taken for blood urea for a year, ranging between 10 and 20 samples every day, and there has in all



cases been reasonable agreement between the creatinine determination and the blood urea determined by Watt and Chrisp's method. All pathological values were controlled by double analysis with the Van Slyke and Kugel method and no clinically significant disagreement was encountered. The only exceptions were patients receiving sulphonamides or P.A.S.

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ADDENDUM

Since this paper has been printed we have learned that W. Daubenmerkl (personal communication) has elaborated a modification of our method. He read the extinction at 435 nm. instead of 420 nm. By reading at this wavelength instead of at the maximum (420 nm), the extinction of the blank value decreases to about 1/7 while the specific extinction (extinction of test solution minus extinction of blank) decreased by only about 40%. Thus it is possible to read the extinction with the test solution against the blank instead of reading both with water as blank, a modification which saves time. Also, this modification makes it possible to read the reaction in a simpler and cheaper type of instrument, *e.g.*, the Bausch and Lomb Spectronic, whereas the reading with our original technique had to be performed in an instrument with a quartz prism and quartz cells. The accuracy of the method becomes much better at the lower range by reading at 435 nm. instead of 420 nm.

The considerable advantages of Daubenmarkl's modification is illustrated by the following table:

CONCENTRATION (MG./100 ML.)

Sample No.	*Van Slyke*	*Watt and Chrisp Reading at 420 nm.*	*Watt and Chrisp Reading at 435 nm.*	*Sample No.*	*Uvispec Reading at 435 nm.*	*Spectronic Reading at 435 nm.*
1	19	30	14	1	28	29
2	41	73	38	2	28	30
3	97	126	102	3	21	24
4	32	50	28	4	29	31
5	97	137	107	5	21	36
6	72	103	70	6	146	141
7	29	38	21	7	126	116
8				8	54	55
9	116	155	133	9	185	178
10	23	26	18	10	312	275
11	27	17	23			
12	48	47	47			
13	36	55	29			

With this valuable modification the method of Watt and Chrisp comes within the reach of laboratories with modest equipment and attains the same grade of accuracy as the other routine laboratory procedures for blood urea besides being much more suitable for routine clinical pathology than the other available methods.

It must, however, be emphasized that extreme care is necessary in preparing the reagents, that high quality distilled water is required, and that fresh reagent (4) has to be used every week.

A method for the collection of saliva

P. J. COUSINS[1] *From the Hospital for Sick Children, Great Ormond Street, London*

The collection of saliva from normal adults and children—unless they are very young—presents no difficulty, but where the patient is unable to use (or, as in one case, considered it rude to spit for any reason) a sputum receiver the collection presents a problem.

During a study of saliva from children with phenylketonuria (Cousins, Philips, Stroud, Wise, and Woolf, 1960), a simple method of collection was adopted. A pasteur pipette with a bulb was cut short and a piece of polythene tubing of 2 mm. bore affixed. The end of the polythene tube was warmed and pushed on to the pipette tip, this having been similarly warmed; on cooling the joint was found to be very firm. The tubing was cut, so that its length was 6 cm., the cut being diagonal across the tube. This end was warmed until pliable and then squashed against a glass plate, thus forming a thickened and wide orifice approximately 4 mm. × 2 mm.

The patient was in one of two positions depending on the degree of cooperation:

(1) The lateral position of the head while lying flat; assistance in holding the head is necessary in cases of bad cooperation.

(2) Normal sitting position; assistance is necessary only in the case of the very young.

The pipette is introduced (*a*) to the cheek wall, and (*b*) under the tongue. Saliva can then be collected by normal pipettage. Amounts of the order of 2 to 3 ml. can be collected in a comparatively short time and transferred to a stoppered test-tube (Exelo 10 cm. × 1 cm. most useful).

The best times for collection are just before a feed or at least two hours afterwards, there being no contamination by food residues at these times.

The advantages of such a pipette are:

(1) Should the patient bite down on the pipette no harm is done, the portion in the mouth being polythene.

(2) No trauma occurs using polythene.

(3) Stimulation of salivary glands is easily effected.

(4) Polythene is easily cleaned and the pipette is easily and quickly cleaned in water.

(5) Such a pipette has a longer life than an ordinary bulb pasteur pipette.

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A rapid method for the estimation of serum haptoglobin

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Since the discovery of haptoglobin by Polonovski and Jayle (1939), variations in the serum concentration of this protein have been observed in a variety of diseases; high levels are found in chronic inflammatory, rheumatic, and neoplastic diseases, and in biliary obstruction whereas haemolytic and hepatocellular diseases are associated with a reduction of the concentration of this protein in the serum (Nyman, 1959).

Methods of estimating haptoglobin depend either upon the peroxidase activity of the haptoglobin-haemoglobin complex in acid solution, or upon the difference in electrophoretic mobility between 'free' and haptoglobin-bound haemoglobin. A simple electrophoretic method is described here which can easily be completed in two hours, and requires less than 0·2 ml. of serum.

MATERIALS

AGAR GEL A solution of 0·8% New Zealand agar in 0·05M *p*H 7·0 phosphate buffer, *i.e.*, Sörensen buffer diluted four-fold.

HAEMOGLOBIN SOLUTIONS For use when normal or low haptoglobin concentrations are anticipated haemoglobin solutions containing 0·2, 0·4, 0·6, 0·8, 1·0, 1·2, 1·4, and 1·6 mg./ml. of carboxyhaemoglobin are prepared by diluting a stock 10 mg./ml. solution with distilled water. Sera with higher haptoglobin contents may be diluted to fall within this range or more concentrated haemoglobin solutions may be used.

BENZIDINE STAIN (Smithies, 1959) Benzidine, 0·2 g., is dissolved in 100 ml. hot distilled water to which 0·5 ml. of glacial acetic acid has been added. This solution is stable for several weeks. Immediately before use 0·2 ml. of 30% w/v H_2O_2 is added.

or

ORTHO-TOLIDINE STAIN (Kohn and O'Kelly, 1955) Mix equal volumes of 4% o-tolidine in ethanol, glacial acetic acid, and distilled water. Add ¼ volume of 5% w/v H_2O_2 immediately before use.

The benzidine stain is more permanent, but the reagent is carcinogenic.

METHOD

Hot agar solution, 12 ml., is poured onto a level glass plate, 11 × 8 cm. When the gel has set a series of holes of approximately 4 mm. diameter is cut with a cork borer. The holes are then filled with the serum-haemoglobin mixtures. These are prepared by adding haemoglobin solutions to equal volumes of serum, using an 0·02 ml. micropipette, giving mixtures containing from 0·2 to 1·6 mg. haemoglobin per 1 ml. of serum. The longer sides of the agar plate are then connected by Whatman 3 MM filter-paper bridges to reservoirs of 500 ml. of the phosphate buffer. A current of approximately 12 milliamps at 240 volts is passed through the agar for 45 minutes, using platinum wire electrodes in

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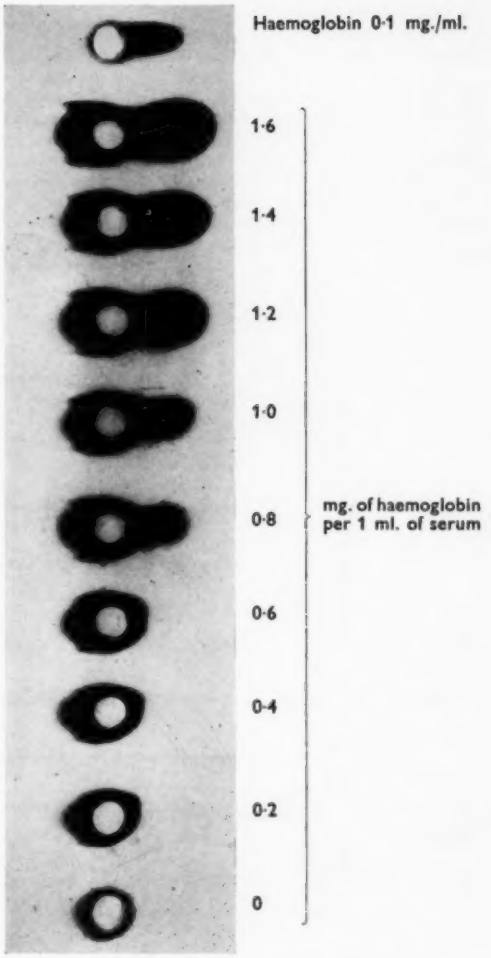

FIG. 1. *Electrophoresis of mixtures of carboxyhaemoglobin with serum in agar gel at* p*H 7·0 stained with benzidine. The haemoglobin-binding capacity of the serum lies between 0·6 and 0·8 mg./ml.*

the buffer reservoirs. The agar plate and the reservoirs are contained within a box to prevent excessive evaporation during electrophoresis.

After electrophoresis the agar plate is immersed in the benzidine stain, which reveals the position of the haemoglobin. Fig. 1, which is a photograph of a portion of a plate, shows that haptoglobin-bound haemoglobin moves slightly towards the anode, and free haemoglobin moves towards the cathode. The binding capacity of the serum haptoglobin in this figure lies between 0·6 and 0·8 mg. of haemoglobin per 1 ml. of serum. Serum to which no haemoglobin had been added shows a small amount of haptoglobin-bound haemoglobin, probably from haemolysis *in vitro*. A solution of 0·01% carboxyhaemoglobin demonstrates that the method is sufficiently sensitive to detect the haemoglobin concentration increments between adjacent cups.

SUMMARY

A rapid electrophoretic method for the estimation of serum haptoglobin is described. Less than 0·2 ml. of serum is required and results are available in less than two hours.

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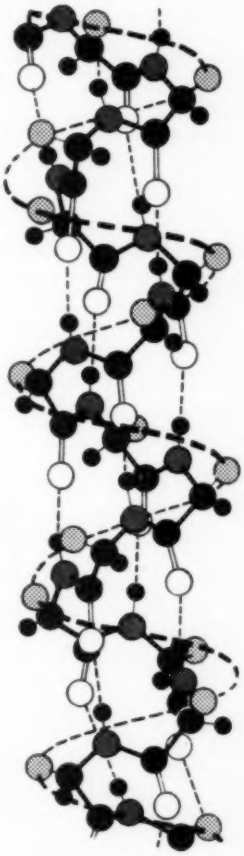

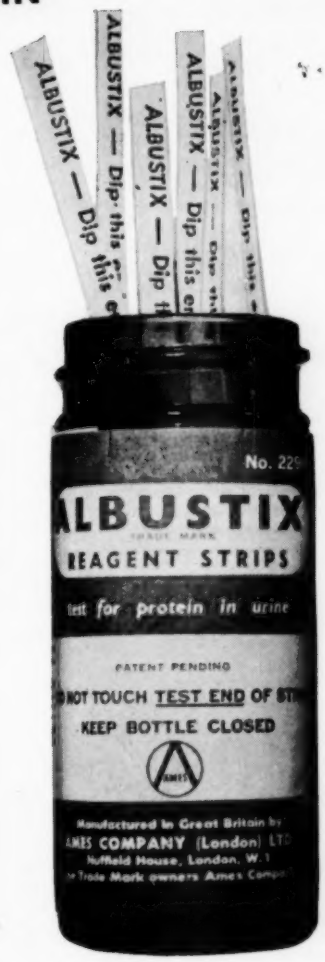

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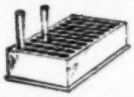

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Symposium on anticoagulant therapy

The management of intravascular thrombosis, particularly that associated with myocardial ischaemia, is a certain source of contention: from the clinical viewpoint there is debate about the criteria upon which anticoagulant therapy is based, especially when it is intended as prophylaxis for heart disease. Even those who select this treatment fail to agree upon its duration; the role of heparin is not fully accepted; and the choice of an oral anticoagulant tends to be empirical and is frequently haphazard.

In the laboratory, the arguments in favour of using the Quick single-stage technique must be balanced against the speed and compactness of the Thrombotest and the undoubted reproducibility of the P and P test.

There does, however, appear to be some glimmer of general agreement that immediate anticoagulant therapy is indicated for the acute myocardial infarction, in which state it seems to lower mortality largely, if not entirely, by reducing the incidence and severity of thrombo-embolic complications; for cerebral thrombo-embolism; for spontaneous and post-operative deep vein thrombosis and pulmonary embolism; and after arterial disobliteration. On the other hand, there is a growing awareness that such therapy should be avoided in treating superficial thrombosis associated with venous varicosity and in all non-embolic cerebral catastrophes.

In the field of prevention, the embolic sequelae of rheumatic heart disease can largely be avoided. Many thoracic and vascular surgeons have reduced their post-operative morbidity by pre-operative prophylaxis. The prognosis in some forms of vertebro-basilar insufficiency, retinal vascular disease, Buerger's disease, and, perhaps, polycythaemia vera, can be improved. In accident and orthopaedic surgery, immediate anticoagulant treatment may considerably reduce the danger of pulmonary embolism. Some cardiological procedures, especially the pre-operative investigation preparatory to mitral valvotomy, can be freed from the risk of thrombo-embolism.

The chief bone of contention is, however, the long-term prophylaxis of myocardial ischaemia. Very simply, the two schools of thought comprise those who believe that oral anticoagulants can prevent or modify reinfarction, and those who do not. They have yet to meet in open contest, though there have been considerable rumblings. Tentative long-range literary exchanges, utilizing statistical ammunition, have inflicted a few casualties, but there is now a sinister new weapon appearing in the form of the symposium. Two international symposia have recently been held and were attended by physicians and surgeons known to favour long-term therapy, and abetted, willy-nilly, by pathologists committed to its supervision whether they favour it or not.

The verbatim proceedings of the second conference, held in London at the house of the Royal Society of Medicine, have been abstracted and are presented here without further annotation, for they contain much of the current debate. Those attending, although sometimes critical and not a little apprehensive, were all actively engaged in some form or other of anticoagulant therapy. Discussion was lively, but no doubt lacked the stimulus of antagonistic criticism. There was a definite balance of opinion in favour of prolonged prophylaxis in heart disease and this was supported by impressive and well-documented large series with control figures for comparison from Great Britain and from Norway and South Africa.

THE ACTION OF ORAL ANTICOAGULANTS

Sir George Pickering (Oxford) took the chair at the opening session, at which the first paper by R. B. Hunter (Dundee) reviewed the action of oral anticoagulants on the blood coagulation mechanism from their discovery to the present day. Oral anticoagulants, influencing both intrinsic and extrinsic coagulation systems, affected prothrombin and factors VII, IX, and X; this was complicated by a parallel alteration of thromboplastin generation. Regarding the problem of haemorrhage, it was reasonable to say that controlled anticoagulant therapy is a safe procedure offering a risk which is certainly less than that from recurrent thrombo-embolism. In the question of the best laboratory control technique, Owren's recently introduced Thrombotest was favoured by Professor Hunter, who agreed with the point of view, stressed by later speakers, that there might be an unrecognized moiety occasionally affected by anticoagulant therapy, possibly a key factor in the development of haemorr-

hagic complications, yet measured by none of the tests to be discussed at the symposium. He reviewed the present knowledge of the therapeutic effect of anticoagulants and the significance of deviations in the intrinsic and extrinsic clotting systems.

In the subsequent discussion, L. Poller (Manchester) drew attention to a depression of thromboplastin generation during phenindione therapy as demonstrated by thromboplastin generation tests routinely performed in parallel with the usual Quick single-stage control. It had been noted that the fluctuation of thromboplastin generation in a high proportion of patients failed to parallel the observed depression of the Quick prothrombin activity; this divergence might be of importance in relation to control and dosage. In three cases with spontaneous haemorrhage, thromboplastin generation was severely impaired, yet in many cases with equally severe depression there was no bleeding: this suggested that anticoagulant therapy interferes with some mechanism, other than those at present acknowledged, which might govern the incidence of haemorrhagic crises.

ANTICOAGULANTS AND THE LIVER

Sheila Sherlock (London) discussed anticoagulants and the liver, and drew attention to the fact that there is a much lower incidence of coronary thrombosis amongst patients with cirrhosis; this may be because liver disease leads to a deficiency of factors V, VII, IX, and X, coupled with a depression of lipoprotein lipase and, in many cases, there may be a platelet deficiency as well. Studies on patients with liver disease showed that oral anticoagulants had no effect on liver function tests but, because of the apparent reduction in clotting in such patients, anticoagulant therapy is seldom necessary. Phenindione tolerance tests were performed on patients with well-compensated liver disease, using first 2 mg. and then 4 mg./kg. body weight in a single dose: the single-stage prothrombin index rose higher and remained elevated longer in these patients compared with subjects without liver disease. Jaundice due to phenindione sensitivity is extremely rare if, indeed, it has ever been correctly reported. In the cases described, it was not possible to assess the particular type of jaundice produced but the pattern of toxicity might be similar to what one would expect with drugs containing a benzine ring.

In the subsequent discussion, it transpired that no member of the symposium had experience of jaundice as a result of anticoagulant therapy nor had any speaker personal knowledge of a case.

H. W. Fullerton (Aberdeen) referred to studies of the recalcified plasma clotting time after a high fat intake. He found that the usual acceleration of this time after three-and-a-half hours failed to occur in patients on phenindione therapy either for recent infarction or on a long-term basis for myocardial ischaemia. A. S. Douglas (Glasgow), commenting on the lower incidence of atheroma in cirrhotics and the coincidental increase of fibrinolytic activity, recognized this to be a feature of importance in assessing prothrombin times in patients with liver disease. He suggested that the fibrinogen level should be studied because of the possibility of intense fibrinolysis. In contrast antihaemophilic globulin is extraordinarily stable in such patients, although this factor is also susceptible to proteolytic degradation.

R. G. Macfarlane (Oxford), speculating on the coincidental depression by phenindione of four apparently dissimilar clotting factors, raised the possibility of a common carrier protein of similar physical properties. He wondered whether anticoagulant drugs might inhibit other forms of protein synthesis, perhaps carried out in the liver. F. Koller (Zurich) remarked on an increase in heparin antithrombin activity in addition to increased fibrinolysis in advanced cirrhosis.

LABORATORY CONTROL OF ANTICOAGULANT THERAPY

There followed two sessions, under the chairmanship of R. G. Macfarlane, dealing with aspects of laboratory control. P. de Nicola (Pavia) outlined the place of the Quick single-stage test, re-evaluating it on the basis of newly discovered factors. The problems concerned with the choice of thromboplastin were mentioned and general directions were given for ensuring safe anticoagulant control. P. A. Owren (Oslo), discussing the laboratory control of anticoagulant therapy, referred to the well-known shortcomings of the Quick single-stage test: that it is too sensitive to variation of factor V and fibrinogen; that the specified calcium concentration becomes susoptimal in the haematocrit if the tested blood is outside the normal range; and that Quick's original method with rabbit brain thromboplastin gives insufficient sensitivity, because of the short range of only seven seconds from the control to the therapeutic level, when using an adsorbed plasma dilution curve. He went on to discuss how these shortcomings had been overcome first by the P and P test and, more recently, by his Thrombotest. The latter was designed to be extremely simple to use and it takes into account depression of prothrombin and factors VII, IX, and X, at the same time providing a suitable reagent for universal use. He stressed that results should always be given as a percentage of normal by the use of a correlation

curve. This should be prepared by serial dilutions of a normal standard plasma in adsorbed normal plasma. The widely adopted practice of diluting normal plasma with saline or buffer results in false prolongation of prothrombin times at higher dilutions, because of alteration of fibrinogen and factor V concentration. Further, neglecting a constant citrate concentration in serial dilutions increases the error. In his opinion, the use of the Thrombotest permitted large-scale anticoagulant therapy control without these drawbacks and without the unnecessary introduction of error into laboratory procedures. He speculated upon the fact that it may be depression of factor IX which is most closely associated with the tendency of a given patient to show haemorrhagic manifestations. In discussing the arbitrary selection of a therapeutic range, Professor Owren referred to a percentage activity based on dilution curves, with an adsorbed plasma diluent. He selected a 10-25% therapeutic range: below 10%, haemostasis became defective, presumably largely due to factor IX depression, and above 25%, he felt that the therapeutic results were not good enough to justify long-term therapy. With the Thrombotest he was maintaining patients at a level much nearer to 10% with perfect safety. He provided figures to show that, in the treatment of venous thrombosis, a level of 15% is highly efficient in preventing further thrombo-embolism in, for example, rheumatic heart disease or post-operative states. As regards arterial disease, he was less confident, but showed results that indicated a highly significant improvement in the re-infarction rate when comparing groups of patients treated within a range below 20% with those maintained at 50% activity. He stressed the importance of maintaining therapy at the maximum intensity consistent with safety. W. Walker (Dundee) then described his experience with Thrombotest, pointing out that recent work had been free of the difficulties earlier encountered with both capillary and venous blood. These difficulties are of the kind that might initially discourage a routine laboratory but can be avoided by observing simple precautions in technique; illustrating this, he outlined the technique in detail and demonstrated advantages of the method, although the testing of venous blood stored overnight is not so straightforward.

T. B. Counihan (Dublin) opened the subsequent discussion and drew attention to difficulties experienced in Eire because so much treatment is based on postal samples. G. A. McDonald (Aberdeen) enquired as to the best site for removing the capillary specimen required for the Thrombotest, and it was generally agreed that the side of the pulp of the finger or the lobe of the ear were best incised by a narrow scalpel blade. One of the drawbacks of Thrombotest, when used as a capillary assessment, is the need for a relatively large amount of capillary blood, and many patients prefer the use of an intravenous needle.

There was some general discussion on the question of a rebound phenomenon following the discontinuance of anticoagulant therapy. No statistical evidence is available to assess this, but there was a general feeling that such a possibility could not be ignored and that anticoagulant therapy should be 'tailed off' in order to cover the possibility of an increased tendency to clot. No suggestion as to how long this might be required was forthcoming. Owren's figures showed that most patients died in the second or third month after discontinuance of therapy and this appeared to be too long a latent period for the rebound phenomenon to be implicated. Experience in Norway showed that the tendency increased with age but that it lasted only a few days.

Rosemary Biggs (Oxford) presented a paper on the laboratory control of anticoagulant therapy, in which she reviewed experience up to date. She drew attention to the fact that for people who were interested in the study of coagulation mechanisms the problem of control presented little difficulty, but where tests were widely employed in routine use by people who had no especial interest, then random variability occurred. She considered the Quick single-stage test to offer a high degree of random variability. She considered Thrombotest probably to be the method of choice for smaller laboratories without the services of individual experts. Her experience with the P and P test led her to believe this to be the best available method of assessment in larger laboratories, for there is much less random fluctuation and a more obvious trend in relation to dose and a greater sensitivity in the near normal range. As this test involves two reagents instead of one, which have to be standardized, it is only reliable when used in laboratories undertaking coagulation studies on a substantial scale.

ANTICOAGULANT TREATMENT AND THE PATHOLOGIST

R. Aidin (Worthing) followed with a paper discussing the position of treatment from the point of view of the pathologist and there followed a lively discussion on the comparative merits of Thrombotest and the Quick single-stage method. Dr. Biggs concluded the discussion with the comment that it was probably better for those who have no primary interest in coagulation mechanisms to use a method already shown to be feasible by the wealth of other

people's experience rather than trying to pit their own lack of experience against insuperable odds.

The last session of the first day, under the chairmanship of H. W. Fullerton, started with a paper by F. Koller (Zurich) discussing his experience with the use of fibrinolysins in the treatment of thromboembolic disease. He reviewed briefly the mechanism of the fibrinolytic system and contrasted it with that of intravascular coagulation. Although antagonistic, these two biological phenomena possess many similar features. Both are dependent upon the final production of a proteolytic enzyme, thrombin or plasmin. In each system the proteolytic enzyme is derived from an inactive plasma precursor, the activation of which can be produced in both systems by two different mechanisms: by a tissue activator or a plasma activator, the latter being generated exclusively by constituents of the blood itself.

Clinical experience was based on the intravenous use of streptokinase as an activator of mechanisms themselves activating plasminogen within the vascular system. An alternative method, by the intravenous infusion of human plasmin itself, probably acts by a similar route because plasmin preparations currently available are contaminated by the streptokinase used in their preparation and it is likely that any therapeutic efficiency is directly proportional to the degree of contamination present. Professor Koller presented a preliminary report on the results of treatment of 103 patients receiving parenteral fibrinolytic activators. Purified streptokinase, plasmin, Pyrexal (a lipopolysaccharide of bacterial origin), nicotinic acid, and Complenin (a combination of nicotinic acid) were used singly. The dose of each preparation was estimated arbitrarily for each patient by assessing the amount of the preparation required to produce dissolution of a clot within 10 minutes: by thromboelastograph methods, considerable individual variation was experienced, but it was found possible to plan a general dose schedule for each patient, who usually received an intravenous infusion over one or two hours. Plasminogen is rapidly consumed and two hours appeared to be an optimal time for maximal activation. Beyond this, further streptokinase has no plasma substrate to act upon but, as plasminogen can be adsorbed on to the surface of the fibrin, streptokinase may still continue to have an effect after plasminogen has disappeared from the plasma. Clinical experience shows that infusions can be continued for 20 or 30 hours. Venous thrombosis of the leg and pulmonary embolism were treated with apparent success, but in arterial thrombosis results were equivocal, for distal infarction areas remained necrotic, irrespective of any clearance of the blocked artery.

Side-effects of this treatment, such as rigors, were not seen when highly purified preparations of streptokinase or plasmin were used. Haemorrhage was prone to occur with the use of higher doses because fibrinogen, factor V, and factor VIII were attacked, in addition to the pre-formed fibrin. The concentration of these factors is not reduced below 10% to 30%, however, and any bleeding tendency is usually mild. Tests *in vitro* to estimate clotting may be vitiated and given a spuriously lengthened time because the breakdown products of fibrinolysis may interfere with the further polymerization of fibrin. Allergic reactions were noted only with less purified preparations and well controlled fibrinolytic treatment was found to produce less haemorrhage than, for instance, heparin therapy.

The safety of intravenous fibrinolysin therapy can be enhanced by the availability of a fibrinolysin antagonist: Σ amino-caproic-acid has been proved to be the most efficient of these agents and is very closely related to the essential amino-acid lysine. It is harmless and can be given in doses of 10 or even 30 g. per day by mouth or a slightly lower dose intravenously. It is well tolerated and, although rarely required in fibrinolytic treatment, it immediately corrects any spontaneous bleeding.

SELECTION OF PATIENTS FOR LONG-TERM ANTICOAGULANT TREATMENT

P. A. Owren (Oslo), in a second paper, discussed the selection of patients for lifelong anticoagulant therapy and described Norway's experience of the organization of large groups of patients. Bearing in mind that anticoagulant therapy is prophylactic rather than curative, he emphasized the value of lifelong therapy in any clinical disease having a high tendency for venous or arterial thrombo-embolic complications. The importance of starting this before the disease is too far advanced was stressed; in any clinical studies so far published the inclusion of advanced cases has largely masked the results of therapy for the rest of the group.

The general structure of a convenient and economical organization is dependent primarily on selection into groups of varying priority. Patients in Norway have been classified into two main groups: a high priority group of patients on whom anticoagulants have a marked effect on mortality and disablement, and a group with second priority where the beneficial effects of anticoagulant therapy are less well established. The high priority group can be divided into five classes. First rheumatic heart disease with embolism; without therapy the frequency of recurrent embolism is high but the effect of therapy is dramatic and results so far raise the

question of whether prophylaxis might start before the first embolic episode, since this is often catastrophic. The frequency of embolism in unselected patients with rheumatic heart disease is 20%-30% over a 10-year period; in a group of 30 patients receiving long-term therapy none has had an embolism over a period of three years. The second class comprises those suffering from angina pectoris of less than two years' duration; here also anticoagulants have had a marked effect. Of 470 receiving long-term therapy, the mortality was only 3·6% per year against the usual mortality in similar patients not treated of 6% to 10%. The third class are patients with one attack of myocardial infarction and, in this large group, patients were selected who had had no angina or had had the condition for less than two years. Results showed that new infarctions were more frequent in patients with more than one infarction before therapy, both in the treated groups and in the control groups: it is safe to assume that anticoagulant therapy is most effective at an early stage of the disease but it does not appear to protect this class of patients to any appreciable extent.

The fourth high priority class in those suffering from atherosclerosis of the legs without signs or symptoms of coronary or cerebral arteriosclerosis; a high proportion of these ultimately died because of coronary disease or some cerebral catastrophe. The mortality rate of 140 such patients treated for four years was 2·7% to 3·7% per year. The fifth and final class receiving high priority suffer intermittent insufficiency of the cerebral circulation, presumably due to thrombosis. Experience in Norway is limited but strongly suggests that these patients should have anticoagulant therapy, a point which did not receive support from subsequent speakers.

The second main group of patients receiving less priority can be subdivided into three main classes: 1, rheumatic heart disease without embolism; 2, angina pectoris of more than two years' duration; and 3, two or more myocardial infarcts. For these classes there is only suggestive evidence that anticoagulant therapy decreases mortality and it does not appear justifiable to insist on treatment.

Professor Owren believed that in Norway probably 1% of the population in the next 10 years will require long-term anticoagulant therapy; he next discussed the problem of handling such a large number of patients using Thrombotest as a measure of control and, finally, indicated that this organization is inexpensive and that the laboratory costs amounted to about £1 per year per patient.

In opening the discussion which followed, M. F. Oliver (Edinburgh) provided figures derived from an analysis of patients discharged from the Edinburgh Royal Infirmary during 1959 following treatment of angina, acute coronary insufficiency, and acute myocardial infarction. From these figures he calculated that for the country as a whole 46,000 patients will start anticoagulant treatment every year. The approximate cost for this number of patients would be £850,000 a year and, if they are going to have lifelong anticoagulant therapy, allowing for the mortality known to occur in people who do not receive it, then some £6m. will be spent every year in giving this form of treatment for coronary disease. By using Professor Owren's selected categories of priority it should be possible somewhat to reduce the cost.

A second point introduced by Dr. Oliver was consideration of the comparatively little protection given to women as opposed to men. Combining the results of the various large-scale trials, it would seem that there is a case for expanding investigations to find out whether women enjoy the same degree of protection as men. In his opinion they do not and, by confining therapy not only to males the number of people involved could be further reduced. By making use of every possible reduction, it would seem that some 13,500 patients would still require attention, and, if they attended every three weeks, this means 4,500 attending hospitals and clinics in Britain each week. The cost of such a service would be considerably in excess of that already allocated for the entire poliomyelitis immunization programme.

Professor Owren (Oslo) drew the attention of the symposium to the tendency to discuss the cost without bearing in mind the parallel considerable saving in both time and money effected by anticoagulant therapy. For example, admissions to hospital could be reduced and patients could return to work much sooner.

ACUTE CORONARY INSUFFICIENCY

On the second day, under the chairmanship of Professor O. Meyer (Wisconsin), P. H. Wood (London) opened with a paper on acute coronary insufficiency. He first defined the condition as an unstable state in which the coronary circulation is insufficient to meet the full metabolic demands in the myocardium under resting conditions but is sufficient to prevent necrosis. There is a background of ischaemic heart disease in at least 80% of cases; the onset is sudden and the patient may become totally incapacitated within 24 hours, still without cardiac infarction. The natural history of the condition shows that cardiac infarction develops in approximately 22% within a period of two months. Indeed this same state precedes about a quarter to

one-third of all cardiac infarctive episodes. The overall mortality is about 25%. Necessarily there are no laboratory data indicating cardiac infarction; the E.S.R. is normal, the temperature is normal, and the serum transaminase and lactic dehydrogenase activity is also normal. One hundred and fifty consecutive cases of subacute coronary insufficiency were studied, 100 being treated with anticoagulants and the other 50 serving as controls. When the effect of treatment became appreciable, it was no longer considered justifiable to continue a control group and all patients received therapy. The rate of infarction occurring within two months of seeing the patient was 3% among those treated and 22% among those untreated. The minimum mortality rate, allowing for untraced cases, was 6% in the treated group and 30% in the untreated controls. As far as their final state of health was concerned, the difference was less noticeable, the treated patients only doing twice as well as the controls. A year or two later these figures showed a clear indication that anticoagulant therapy must be given when a patient has acute coronary insufficiency thought to be due to thrombosis. Treatment should start with heparin and phenindione given simultaneously.

EVALUATION OF LONG-TERM ANTICOAGULANT TREATMENT

A. S. Douglas (Glasgow) dealt with the results of the Medical Research Council's trial on long-term therapy. The trial had now been completed but it was permissible for him to speak only of results from the time of the first entry of cases to the trial in November 1955, up to the time of the initial publication of the report in March 1958. He commented upon some of the criticisms which had been directed at this trial and enlarged on some of the difficulties which had been encountered. As regards treatment of women, numbers were too small in the M.R.C. trial to gain adequate evidence, but by assessing the three trials which have had a similar design there had been no demonstrable difference in mortality in females and only a borderline improvement in the rate of infarction. Dr. Douglas had also calculated the requirements of long-term anticoagulant therapy: the incidence of patients who have suffered an infarction within the last two years and are still alive is 1 in 380 and the incidence of patients suffering from angina is 1 in 170; these figures are based on a survey of an area of west Scotland covering a population of 70,000, and the overall incidence of patients in both these categories approximated 1 per 100.

M. M. Suzman (South Africa) presented a paper on the evaluation of the effect of long-term anticoagulant therapy on the prognosis of coronary artery disease. This had involved a considerable amount of work and included documented observations on more than 2,000 patients. It was shown that mortality rates in survivors of myocardial infarction maintained on long-term treatment are consistently lower than the rates observed in survivors whose treatment with anticoagulants was limited to the acute phase, and this difference in death rate was observed in all age groups. More than 3,000 patients in the long-term group of treated cases were compared with 4,800 in the entire short-term group, the five year survival rate being a little over 50% in the untreated group and about 75% in the treated group. A history of infarction before the presenting attack materially increased the mortality in both groups of patients but to a far greater degree in those not receiving long-term anticoagulants. Amongst patients who discontinued long-term treatment, the subsequent mortality rates were higher than in those who maintained treatment without interruption.

In the subsequent discussion C. S. McKendrick (Liverpool) and K. Ball (London) referred to the problem of handling patients in large numbers, and their remarks were echoed by many other speakers. Dr. Ball referred to the observations in the M.R.C. study that both deaths and recurrence effects were most apparent in the first three months; as a result he had adopted the practical compromise of placing patients suffering from their first infarct on anticoagulants for three months after they leave hospital (a total of four months altogether) and then tailing off therapy over the next six weeks.

A. Morgan Jones (Manchester) criticized the use of the term 'acute coronary insufficiency' as being neither a pathological entity, such as thrombosis, nor a symptom, such as angina. He recalled that ischaemic heart disease is known to be a phasic disease with periods of activity and periods of quiescence, the former lasting anything up to 18 months and the latter 10 or more years. He suggested that cases should be differentiated and that anticoagulants in the first instance should be limited to those known to be suffering from the active phase of the disease. He speculated that the better results shown by Dr. Paul Wood's study were partly due to the fact that all his patients by definition were in the active phase; the poorer results of the longer M.R.C. trial might be due to the fact that only a small proportion of patients after a myocardial infarct remain in the active phase. S. Sevitt (Birmingham) felt that there could be no doubt about the value of short-term anticoagulant therapy in myocardial infarction. He stressed that the beneficial effects were not necessarily related to the underlying cardiac condition but a high proportion

of patients were saved who would otherwise have died, because venous thrombosis and pulmonary embolism had been prevented. In this respect it was important to know what the incidence was of recent intravascular coronary thrombosis amongst those who died in a treated series compared with the incidence in those who died without anticoagulant treatment.

R. L. Richards (Glasgow) described the indications for anticoagulant therapy in peripheral arterial disease, emphasizing that clinical success can be gauged only by prophylaxis and not from any curative effect. Anticoagulant therapy has an important place in the management of acute and chronic arterial occlusion, especially of peripheral vessels, and as an adjunct to disobliterative surgery. J. L. Stafford (London) illustrated the experience gained in the organization of a large out-patient clinic covering long-term anticoagulant therapy in a teaching hospital. He drew attention to the hidden costs incurred by the transport of patients to and from clinics and the mounting expenditure of professional and technical time devoted in the laboratory to this commitment.

Sir W. M. Morrow (Australia) spoke on the problem of long-term anticoagulant therapy in the country districts of Australia and described the difficulties facing general practitioners having minimal laboratory facilities. D. Stark Murray (Kingston) opened the discussion by referring to his own experience in running a domiciliary service in his own region where the majority of patients were nursed at home; he described a bedside technique developed for the control of anticoagulant therapy which seemed to work satisfactorily. But he also stressed the cost of the service in both time and money and the impact of this commitment on the other services rendered by a clinical pathology laboratory.

E. Petrie (London) described the general practitioner's role in anticoagulant therapy and his own endeavours to control treatment, utilizing a modified prothrombin activity assessment which he carried out himself. S. Sevitt (Birmingham) presented a paper on anticoagulant prophylaxis against venous thrombosis and pulmonary embolism in injured patients, and drew upon his considerable experience at the Birmingham Accident Hospital. It was now their accepted practice to advocate prophylactic anticoagulant therapy for all patients who were considered to be at risk of venous thromboembolism. Indeed, it was the rule to provide this treatment, unless there was a contraindication, for all those over the age of 45 and admitted for a period of bed-rest predicted to be longer than three or four days. Figures were presented to indicate that the incidence of pulmonary embolism had been reduced by as much as 80% or 90%.

ANTICOAGULANT TREATMENT OF CEREBRAL VASCULAR DISEASE

C. H. Milliken (Rochester, U.S.A.) presented evidence from the Mayo Clinic on the use of anticoagulants in cerebral vascular disease. He stressed that such treatment was strictly limited to a few categories of patient and these, although important, comprise only a small proportion of patients with cerebral disease. He eschewed long and complicated classifications and preferred to use the simple term 'stroke'. Of patients suffering a primary cerebral episode there are three main groups: 1, Those with incipient or impending stroke in whom neurological examination is generally normal, but there may be some warning indicating that a stroke is likely; 2, advancing stroke in patients suffering an active progression of neurological deficit, during the period of observation extending over a matter of hours; and 3, those with a completed stroke where the neurological deficit, although of variable degree, is stable but may be followed by improvement over days or months. Of these three conditions only the first should receive anticoagulant therapy; it is convenient to make a distinction between the two vascular systems involved in this category. First, because there is a difference in mortality until ultimate thrombosis and, secondly, because there is a difference in the incidence of haemorrhage in the two systems with or without anticoagulant therapy. Thus one speaks of intermittent insufficiency in either the vertebral-basilar or the carotid systems. Selected cases with advancing thrombosis in the vertebral-basilar system might also benefit from therapy if the diagnosis is distinct. Apart from these sharply defined and strictly limited categories, only the recurrent embolic phenomena associated with cardiac pathology are suitable for anticoagulant therapy.

J. Marshall (London) also dealt with the use of anticoagulants in cerebrovascular disease, outlining the difficulties in diagnosis, especially in distinguishing haemorrhage from infarct. He considered that the indications for anticoagulant therapy at the present time are limited, first to those with acute cerebral infarction due to embolism; secondly, those with known embolic infarction if seen at a stage at which development is slow and still progressing; thirdly, in patients experiencing recurrent ischaemic episodes in the vertebro-basilar territory; and

fourthly, in patients prone to have a partial stenosis of the internal carotid artery which is giving rise to symptoms.

The final paper by R. W. Emanuel (London) was on the treatment of pulmonary hypertension and mitral valvular disease in relation to anticoagulants. This field of clinical study had received little attention. Experience at the National Heart Hospital and Brompton Hospital was based on four classes of patient. In the treatment of mitral stenosis complicated by severe pulmonary hypertension it was found that anticoagulants protect patients from fatal thrombo-embolism while waiting for cardiac catheterization and operation. In mitral stenosis uncomplicated by pulmonary hypertension, anticoagulants appear to diminish the risk of systemic emboli. Subacute thrombo-embolic pulmonary hypertension can be cured if anticoagulants are used sufficiently early, but this form of treatment appears to have no effect on the natural history of primary pulmonary hypertension and, indeed, may cause fatal haemorrhage in cases of the Eisenmenger syndrome.

During the final session of the symposium a panel discussion was held during which time questions presented during the meeting were answered extemporaneously. Professor Sir George Pickering (Oxford) took the chair and his fellow members were Professor R. B. Hunter (Dundee), Dr. C. H. Milliken (Rochester, U.S.A.), Professor P. A. Owren (Oslo), and Dr. P. H. Wood (London).

The first question from Dr. Sevitt was, 'It is now established that effective oral anticoagulant therapy for cases clinically diagnosed as having deep vein thrombosis of the lower limb reduces the incidence of fatal pulmonary embolism from 80% to 90%. Why is this so? Why is it that anticoagulant therapy seems to prevent detachment of the clot?'

Some surprise was expressed that the rate could be cut down by as much as 80% to 90%. It was suggested that efficient anticoagulant therapy functioned by reducing the tendency for the friable end of the clot to break off. Secondly, the prevention of further extension of the thrombus is bound to reduce the possibility of complications. The size of the embolus is reduced; it is probably possible to reduce the mortality of pulmonary embolism by 90% but unlikely that the incidence can be reduced by such a considerable figure.

Dr. R. G. Macfarlane (Oxford) enquired, 'Should a pathologist or a clinician, or both, control anticoagulant therapy?' This question evoked a great variety of personal opinions which, on the whole, reflected the specialty of the person commenting. Generally speaking clinicians, particularly physicians, preferred to maintain their own clinical control, but accepted the necessity for laboratory surveillance, and, with it, the collaboration of the clinical pathologist.

The development of this form of hospital control in Britain has been haphazard and many people would appreciate guidance on how best to deal with the problem. At the moment, however, many clinicians remained disinterested and it was better, in such circumstances, for the clinical pathologist to assume control, so long as he maintained a close liaison with both the physician and the general practitioner.

The introduction of the Thrombotest might make it possible for a clinician relatively inexperienced in laboratory methods to maintain an anticoagulant clinic in the way that the hospital diabetic clinic has been organized; this possibility, with the implication that technical staff would work directly under the control of the practising physician, has yet to be explored. There is some difference between a general hospital and a teaching hospital, where highly specialized laboratory facilities may be more readily available. This question of the responsibility of clinical supervision of groups of patients raised a matter of considerable importance regarding the relationship of the clinical pathologist and his colleagues.

Professor Counihan (Dublin) asked, 'What is the size of risk of subintimal haemorrhage in coronary atheroma when anticoagulants are begun? Is there a period of time when this risk is much greater in relation to a clinical episode of coronary occlusion?'

There appeared to the Panel to be no direct answer possible, as the presumption of subintimal haemorrhage leading to coronary occlusion has never been adequately confirmed. If it occurred at all frequently it was felt that many would have had the experience of a patient in a state of acute coronary insufficiency developing massive occlusion under therapy, yet this in fact is very unusual. It was considered that such a risk was not serious if indeed it existed.

Dr. D. G. Chalmers (Cambridge) enquired, 'Does the Panel feel it necessary to administer phenindione in divided daily doses to patients on long-term therapy?' Opinion was evenly divided. From the scientific point of view there was no necessity to divide the daily dose so far as stability was concerned. Some were in favour of a single dose to minimize the patient's tendency to forget; some felt it better to divide the dose so that, in the unlikely event of trouble, it was easier to correct an overdose. The administrative convenience of a single daily dose appeared to be of greater benefit and was adopted by most large clinics using phenindione. It was generally agreed that the use of divided daily doses

of the long-acting coumarin derivatives is entirely unnecessary.

Dr. S. H. Davis (Edinburgh) asked, 'In view of the increasing use of long-term anticoagulant drugs, would it not be advisable for all hospitals to issue a nationally agreed standard instructional pamphlet and to incorporate in it a page for the inclusion of the type of drugs used, dosage and control data. If so, would the Panel decide the detailed contents?' After consideration and a somewhat humorous discussion, it was generally agreed by the Panel that written instructions must be given to the patient and that it is administratively desirable to provide written information on the nature of the drug, the dose, the patient's blood group, and the address and telephone number of the hospital assuming control. It was widely recognized that no two centres and no two individuals would ever agree on the wording of an instructional pamphlet and it was considered to be undesirable for any such communication to be the product of any central authority. Although it was desirable for instructions to be available, it was agreed that no pamphlet should be designed to take the place of the clinical interview between the doctor running the anticoagulant clinic and the patients coming under his supervision. On the whole, the possibility of a pamphlet proving satisfactory to all concerned appeared to be quite remote. Patients who were mentally incapable of cooperating with the written instructions were possibly better avoided in the first instance.

Professor Koller (Zurich) asked, 'How long should one continue to treat pulmonary embolism in order to avoid cor pulmonale?'

It was felt that anticoagulant therapy must be continued until the pulmonary vascular resistance is normal. In the case of a young man with an efficient dual circulatory system, this might be effected within six to 12 weeks; on the other hand, an elderly person with recurrent embolism may have to receive therapy for life and, between these two extremes individual variations were considerable. For a major pulmonary infarction nine to 12 months' therapy is probably necessary. Otherwise the patient may pass into a period of ill-health, which may last a year or more, and which is ascribed to pulmonary hypertension.

The Panel had no useful comment to offer as to whether acute coronary thrombosis occurred at any specific time of the day or week. On the question of the use of anticoagulants for cerebral embolism, there was disagreement. Some members of the Panel felt that anticoagulants, both heparin and phenindione, should be started immediately the diagnosis is made, but this might be followed by an increasing bleeding into the infarction and this danger was present for 24 to 72 hours after the initial occlusion. With this in mind, some workers withhold anticoagulants for at least four days, giving them ultimately with the philosophy of preventing the next embolic event rather than materially changing the infarct which has already occurred. The majority of the Panel favoured abandoning the use of heparin, but one member, on the contrary, felt that any form of anticoagulant therapy was indicated immediately, because most cases of cerebral embolism involved multiple emboli; the maximum disability is reached at once and there are grounds for treatment as quickly as possible.

The next question asked the opinion of the Panel about the administration of heparin in cases of cardiac infarction while awaiting the action of oral anticoagulants. Although there is no statistical evidence one way or the other, all were agreed that heparin should be started at once and continued for at least 24 hours.

Dr. A. G. W. Whitfield (Birmingham) asked about the advantages and disadvantages of long-acting anticoagulants compared with phenindione. The Panel agreed that it is best in any given centre to use the preparation to which those in charge of therapy have become accustomed. This differed from area to area and from country to country. Phenindione is the most popular because of its rapid action and is easier to control on an outpatient basis. Thus, in Europe, the slower acting coumarin derivatives are generally used only for cases where phenindione proves unsuitable.

As outpatient therapy is likely to increase, there will be a substantial rise in the number of patients requiring an alternative form of therapy and it is advisable for clinicians to gain experience in the use of at least one preparation in addition to phenindione. Of those quoted, warfarin sodium or phenylpropylhydroxycoumarin appear to be the most popular.

On the question of switching from one preparation to another, it was difficult to be dogmatic, but it is possible to transfer from the quick-acting phenindione to the slower-acting preparation without delay and this is the course usually required; the change from a slow-acting to a swift-acting preparation runs the risk of potentiation and must be controlled carefully.

The Panel agreed that the treatment of haemorrhagic complications of anticoagulant therapy was best effected by discontinuing the drug for a day or two; scarcely ever should it be necessary to give vitamin K_1 because of the possibility of a rebound coagulative tendency. The accumulated experience of many centres has shown that haemorrhagic complications are much less important than was hitherto appreciated and that it is perfectly safe

merely to stop the drug and wait to see what happens. The one exception seems to be haemorrhage into the eye, but such complications are extremely rare. Selection of the patients before admitting them for long-term anticoagulant therapy should reduce the possibility of dangerous bleeding.

The last question by Dr. A. G. Signy (London) was, 'Would the Panel advise in cases of sensitization to an oral anticoagulant a short period of dose reduction before changing over to a newer drug; under what conditions would the Panel change the drug?' The Panel agreed that sensitization was uncommon and was usually shown by a rash or, occasionally, diarrhoea. Most people switched directly and in comparable doses. With regard to phenindione, some of the sensitivity may be associated with the benzine ring in this preparation.

Broadsheets prepared by the Association of Clinical Pathologists

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. R. B. H. Tierney, Pathological Laboratory, Boutport Street, Barnstaple, N. Devon,** price 1s. each.

1 The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics. 1952. R. W. FAIRBROTHER.

2 Determination of Sensitivity of *M. tuberculosis* to Streptomycin. 1952. R. L. VOLLUM.

3 The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents. 1953. L. C. NICKOLLS.

4 The Estimation of Carbon Monoxide in Blood. 1953. D. A. STANLEY.

5 The Identification of Reducing Substances in Urine by Partition Chromatography on Paper. 1953. G. B. MANNING.

6 The Paul-Bunnell Test. 1954. R. H. A. SWAIN.

7 The Papanicolaou Technique for the Detection of Malignant Cells in Sputum. 1955. F. HAMPSON.

8 Investigation of Haemorrhagic States, with Special Reference to Defects of Coagulation of the Blood. 1955. E. K. BLACKBURN.

9 Daily Fat Balance. 1956. A. C. FRAZER.

10 Mycological Techniques: (1) Collection of Specimens. 1956. R. W. RIDDELL.

11 Mycological Techniques: (2) Cultural Isolation. 1956. R. W. RIDDELL.

12 Techniques for Demonstrating L.E. Cells. 1956. J. V. DACIE and L. S. SACKER.

13 The Identification of Serotypes of *Escherichia coli* Associated with Infantile Gastro-enteritis. 1956. JOAN TAYLOR.

14 The Determination of Serum Iron and Serum Unsaturated Iron-binding Capacity. 1956. ARTHUR JORDAN.

15 The Estimation of Faecal 'Urobilinogen'. 1957. C. H. GRAY.

16 Preservation of Pathological Museum Specimens. 1957. L. W. PROGER.

17 Cultural Diagnosis of Whooping-cough. 1957. B. W. LACEY.

18 The Rose-Waaler Test. 1957. C. L. GREENBURY.

19 The Laboratory Diagnosis of Fibrinogen Deficiency. 1958. R. M. HARDISTY.

20 Investigation of Porphyrin/Porphyria. 1958. C. RIMINGTON.

21 Quantitative Determination of Porphobilinogen and Porphyrins in Urine and Faeces. 1958. C. RIMINGTON.

22 Investigation of Haemolytic Anaemia. 1959. J. G. SELWYN.

23 The Dried Disc Technique for Bacterial Sensitivity Tests. 1959. R. W. FAIRBROTHER and J. C. SHERRIS.

24 Safe Handling of Radioactive Tissues in the Laboratory and Post-mortem Room. 1959. R. C. CURRAN.

25 Titration of Antistreptolysin O. 1959. H. GOODER and R. E. O. WILLIAMS.

26 The Periodic Acid-Schiff Reaction. 1959. A. G. E. PEARSE.

27 The Laboratory Detection of Abnormal Haemoglobins. 1960. H. LEHMANN.

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Book Reviews

DAS INNERE LYMPHGEFÄSZSYSTEM DER ORGANE. By F. Renyl-Vamos. (Pp. 447; 278 figures. Price not stated.) Budapest: Akademiai Kiado. 1960.

The injection techniques by which nineteenth century anatomists worked out the main systems of lymphatic drainage are unsuitable for such organs as the kidney; besides filling lymphatic capillaries, the injection material leaks into tissue interstices and blood vessels and gives a totally misleading picture. Following up an earlier discovery, the author of this monograph found that if he ligated the renal lymphatics at the pelvi-ureteric junction, or indeed the ureter itself, the resulting dilatation of the lymphatics within the kidney made them easily recognizable in sections prepared a few days later. The principle is to produce local oedema, so that the lymphatics become distended by the protein they have to carry away.

The technique has produced several interesting results. Infections in the kidney and ureter and in other organs, including the gall-bladder and uterine tube, spread not via lymphatics but directly through interstitial tissue: leucocytes are never seen inside lymphatic capillaries. In acute glomerulonephritis there is interstitial oedema but the lymphatic dilatation which accompanies it in other conditions is absent and protein stagnation provokes the laying down of collagen fibres.

These views are illustrated by many clear and convincing photographs, but the coloured illustrations in the copy received are indecipherable because the blocks were out of register. The text is easy to read, but is repetitive and contains much irrelevant matter. This work deserves attention from the pathologist interested in lymphatics, but not all the author's views will command universal agreement.

D. P. WINSTANLEY

PROTIDES OF THE BIOLOGICAL FLUIDS: Proceedings of the Seventh Colloquium, Bruges, 1959. Edited by H. Peeters. (Pp. 420; 210 illustrations and 98 tables. 76*s*.) Elsevier Publishing Company, Amsterdam, London, New York, Princeton, 1960.

Every year D. H. Peeters puts those who are interested in the proteins of the body fluids more greatly into his debt. Once again he has edited the papers and discussions given at the annual Bruges Colloquium (the one in 1959 was the seventh of the series). At this Colloquium even more papers, covering an even wider field than previously, are recorded in an excellently produced volume.

There is a first-rate brief review on 'The Synthesis of Antibodies and of Protein' by Professor H. E. Schultze. This is followed by sections on technical aspects of the subject of protein analysis by electrophoresis and spectrophotometric methods. Then come sections on immunoelectrophoresis, on chromatography, on certain biochemical aspects, and on some binding properties of proteins. Then follow sections on nutrition and pathology.

There are very many excellent papers included in the 87 given at the meeting. This volume can be very strongly recommended to all those interested in the body proteins.

J. N. CUMINGS

OXOSTEROIDS: THE USE OF PHENOLIC HYDRAZIDES FOR DETECTION, CHARACTERIZATION AND ESTIMATION. By Bernard Camber. (Pp. viii + 79; 8 figures. 12*s*. 6*d*.) London: H. K. Lewis. 1960.

This is a specialized work. In a field bedevilled by a confusing literature the author conveys a clear picture. The methods described will be of interest both to those who seek to determine these compounds in body fluids and tissues and to those seeking to characterize them in histochemical preparations.

ARTHUR JORDAN

AN INTRODUCTION TO HUMAN BLOOD GROUPS. By Fulton Roberts. (Pp. 85. 9*s*. 6*d*.) London: Heinemann. 1960.

This monograph of 13 short chapters attempts to give a sound basic knowledge of blood groups to those having neither the time nor the inclination to explore the numerous larger volumes filled with curious symbols.

There is a short serological introduction and the author goes on to give a commendably clear account of the rhesus blood groups, their discovery, detection, and importance. Understandably he uses the Fisher CDE nomenclature, although he voices the recent doubt of its validity.

The minor blood groups are dealt with briefly. The ABO group is dealt with later because it displays unusual features, such as the presence of natural antibodies.

Short chapters follow to explain the importance of blood groups in disease and in the study of genetics and anthropology and, finally, in a chapter entitled 'Some Growing Points', current research is discussed. A short bibliography is given.

The author has succeeded in writing a most readable introduction to human serology which can be confidently recommended to students and junior pathologists.

R. H. B. PROTHEROE

BLOOD TRANSFUSION: A GUIDE TO THE PRACTICE OF BLOOD TRANSFUSION WITHIN HOSPITALS, 2nd ed. By George Discombe. (Pp. 58. 6*s*.) London: Heinemann. 1960.

This pamphlet first appeared in 1955 as a guide to the practice of transfusion within hospitals. It is written primarily for the temporarily registered doctor during his first resident appointment. It is, however, popular with students and makes useful reading for the resident pathologist.

The second edition shows some rearrangement and incorporates some advances in knowledge. The development of transfusion is related, and the workings of a regional transfusion centre are described. The difficulties and dangers of transfusion are stressed, but no more of the theory or technique of transfusion is described than is necessary to aid the resident's clinical judgment.

This pamphlet, together with the Ministry of Health publication 'Notes on Transfusion', will greatly facilitate the task of the blood transfusion officer.

R. H. B. PROTHEROE

MICROCHEMICAL METHODS FOR BLOOD ANALYSIS. By Wendell T. Caraway. (Pp. xi + 109. 42*s*.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1960.

This book sets out to present a system of micro-analysis based on the use of the Coleman Jr. spectrophotometer and flame photometer.

It is written as a biochemistry cookery book with the assumption that the cook has not yet even learnt how to light the gas.

Several macro-methods are described and others are somewhat out-dated macro-methods adapted for micro-analysis.

This book would have its place as a free issue with a Coleman Jr. spectrophotometer but can hardly be recommended at 42*s*.

W. P. STAMM

INTERNATIONAL CONGRESS OF NEUROLOGY

The Seventh International Congress of Neurology will be held in Rome from 10 to 15 September 1961, under the auspices of the World Neurologic Federation and of the National Institute for Nervous Diseases and Blindness of Bethesda.

Full details are available from the President of the Congress, Prof. Mario Gozzano, or from the Secretary General, Dr. Giovanni Alemà, Viale Università 30, Rome.

The maturation and senescence of erythrocytes

A symposium was held at the Institute of Chemical Physiology, Humboldt University, Berlin, on the occasion of the 250th anniversary of the Charité Hospital and the 150th anniversary of the University.

Amongst the subjects discussed were:—

THE CYTOCHEMISTRY OF RED CELL MATURATION, contributed by Bo Thorell (Stockholm), who used microspectrophotometry to quantitate and localize intracellular nucleic acids, proteins, haem, and iron, in single marrow cells as well as the loci of respiratory enzymes and coenzymes in certain other cells.

ENZYME CHANGES IN RED CELL MATURATION, SENESCENCE, AND CONSERVATION was discussed by S. Rapoport. He asserted that reticulocyte maturation involved interlinked and serially governed metabolic and structural processes, sharing many pathways with senescence but differing from the changes accompanying storage. The key process in maturation is the decline in respiratory activity caused by protein inhibitors which act on iron flavoproteids, cytochromoxidases, and SH-containing hexokinases and pyrophosphatases. Perhaps the limiting factor for all survival *in vitro* and *in vivo* is the maintenance of adenosine triphosphate levels.

T. A. J. Prankerd (London) stressed the importance of changes in the stroma and lipids in these processes, and C. W. Löhr and A. P. Waller (Marburg) reported on enzyme changes in red cells during senescence *in vitro*.

BLOOD PRESERVATION AND ADENINE NUCLEOTIDES was discussed by H. Yoshikawa (Tokyo), who showed that survival of stored red cells is greatly prolonged by adding adenine to acid synthesis of adenosine triphosphate and inosine for preferential oxidation, sparing the heavy A.T.P. consumption of the glucose cycle. The steps adenine to A.T.P. were delineated and the influence of A.T.P. on red cell morphology was shown. Dr. Fischer (Frankfurt) reported work leading to similar results.

H. Lehmann (London) covered recent advances in the haemoglobins, their chemistry, characterization, identification, distribution, and frequency. He spoke of experiments in hybridization conducted *in vitro* and postulated an interrelationship between the prevalence of abnormal haemoglobins and certain diseases in African communities. The sickle cell haemoglobin confers some resistance to malaria in early childhood.

The proceedings will be published shortly and a further symposium is planned for two or three years hence.

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A clinicopathological study of benign Hodgkin's disease

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From the Department of Pathology, Postgraduate Medical School, Ducane Road, London

SYNOPSIS A series of 44 cases of benign Hodgkin's disease is reported. The condition occurs most commonly between the ages of 15 and 35 years and affects men more often than women. The presenting symptom is painless lymphadenopathy, frequently in the neck, and often confined to a single group of nodes. The histological features are the presence of Sternberg-Reed cells in a background of mature lymphocytes intermingled with a small number of reticulum cells. Mitotic figures are usually present only in the Sternberg-Reed cells. The prognosis is good; 93% of cases survive five years and 85% 10 years. About one-quarter of the cases show transition to classical Hodgkin's disease after a period of five or more years and die with widely disseminated Hodgkin's disease. Biopsy-excision followed by radiotherapy is the treatment of choice.

In 1952 one of us reported six cases of benign Hodgkin's disease (Harrison, 1952). We believed that it was a recognizable histological entity but were then uncertain as to its behaviour. In particular, we did not know to what extent the ultimate prognosis was better than that of classical Hodgkin's disease, especially if the latter was seen and treated in an early stage. Since 1952 we have had the opportunity of studying a considerable number of cases, largely thanks to the kindness of our colleagues. In the present paper we have attempted to define the histological features more closely and to determine whether the separation of this group is justified in terms of ultimate prognosis.

NOMENCLATURE

Jackson and Parker (1944), who first described the condition, called it 'Hodgkin's paragranuloma'; Robb-Smith (1947) described an apparently similar condition under the name of 'lympho-reticular medullary reticulosis'. Lumb (1954) has used the title 'reticular lymphoma' and Symmers (1958) has suggested 'indolent Hodgkin's disease'. In the present paper we propose to use the name 'benign Hodgkin's disease' which we coined in our earlier work. (The term 'classical Hodgkin's disease' will be used for purposes of clarity for the widely recognized and more usual form of the disease which some call Hodgkin's granuloma.)

LITERATURE

Rosenthal (1936) first presented a histological classification of Hodgkin's disease based on the proportion of lymphocytes to reticulum cells. He found that a higher proportion of lymphocytes correlated with a longer survival. Jackson (1937, 1939) and Jackson and Parker (1944, 1947) first defined the condition which they called paragranuloma and showed that the prognosis was clearly better than that of ordinary Hodgkin's disease. Since then, various workers have described series of similar cases (Robb-Smith, 1947; Wright, 1956, 1960; Bonenfant, 1954; Lumb, 1954; Trautmann, 1954, 1959; Marshall, 1956; Symmers, 1958). There is general agreement on the broad definition of the histology and on the improved prognosis. In spite of this, the actual cases remain difficult diagnostic problems. We hope to be able to define the diagnostic criteria a little more closely and to show that the prognosis is so strikingly better that an accurate diagnosis is of great clinical importance.

MATERIAL

We have collected all the examples of possible benign Hodgkin's disease, both from our own hospital and from cases sent in by our colleagues. These were examined without any knowledge of the

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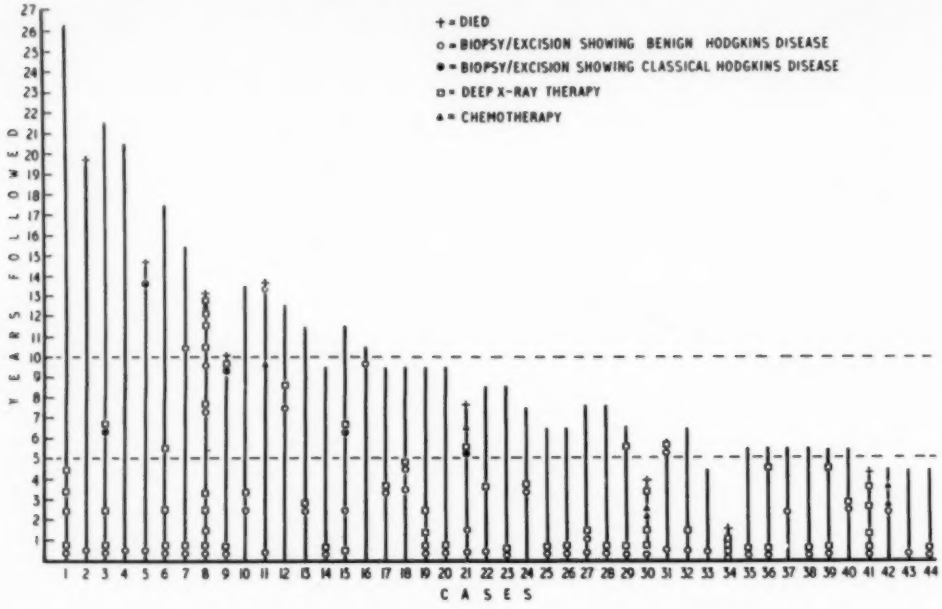
+ = DIED
o = BIOPSY/EXCISION SHOWING BENIGN HODGKINS DISEASE
• = BIOPSY/EXCISION SHOWING CLASSICAL HODGKINS DISEASE
□ = DEEP X-RAY THERAPY
▲ = CHEMOTHERAPY
YEARS FOLLOWED
CASES

FIG. 1. *Histogram summarizing the duration, forms of therapy, recurrences, and follow-up in all 44 patients.*

clinical course. Forty-five cases were found which, on purely histological grounds, we considered to be examples of benign Hodgkin's disease and a follow-up was obtained on these. This was successful in all cases except one, although we have good reason for believing the patient to be alive seven years after the onset of the disease. Because of the long course of the disease, there seemed little point in considering cases of very recent onset, so that patients who had suffered from the disease for less than three years were excluded.

There were then 44 cases of benign Hodgkin's disease selected on morphological grounds with a minimum follow-up period of three years; in fact, all were followed for four years or longer. Fifty-six biopsies from these cases were available for study. Multiple biopsies were available in 10 patients; five of these showed transition to classical Hodgkin's disease. One further biopsy also showed benign Hodgkin's disease, but was too poor technically for detailed study. We were unable to obtain the first biopsy in Case 7; it was reported elsewhere as Hodgkin's disease and because the second biopsy showed typical benign Hodgkin's disease we have presumed that the first was similar and, although marked on Fig. 1, it is not, of course, included in our analysis. A further four biopsies showed normal lymphoid tissue only and are not shown in Fig. 1. The cases are numbered and successive biopsies of the same patient designated by the letters A, B, and C.

Because of the rarity of the condition and because many of our cases were sent to us for an opinion by other pathologists, only paraffin-blocked material was available, and in some cases only a single haematoxylin-and-eosin-stained section was obtainable. Silver-impregnated reticulin preparations were made wherever possible and a few other special stains, notably methyl violet for amyloid, were used to clarify specific points. All histological examinations were made without knowledge of the subsequent course of the patient.

In order to clarify the boundary between benign and classical Hodgkin's disease a control series of 43 biopsies from 42 consecutive cases of classical Hodgkin's disease seen in the department from 1950 to 1955 were also studied.

CLINICAL FEATURES

Details of the presenting features are summarized in Table I. The age of onset varied from 6 to 70

TABLE I

CLINICAL DETAILS

No. of Case	*Age at Onset*	*Sex*	*Sites of Disease when First Seen*
1	24	M	Neck (R), axillae, groin (L)
2	57	F	Neck (L), neck (R)
3	19	M	Axillae, groins
4	12	M	Neck (R)
5	15	M	Neck
6	31	F	Neck (L)
7	18	F	Neck (L)
8	12	M	Neck (R)
9	33	M	Neck (R)
10	16	M	Neck (bilateral), axillae
11	22	M	Neck (L), axillae
12	21	M	Neck (L)
13	33	M	Axilla (R)
14	9	M	Neck (R)
15	6	F	Neck (L)
16	23	F	Mediastinum
17	20	M	Neck
18	29	M	Neck
19	30	F	Neck (L), mediastinum
20	15	M	Neck (L)
21	47	M	Neck (L), groin (R)
22	18	M	Axillae
23	29	M	Axilla (R)
24	58	M	Neck (R)
25	21	M	Neck
26	25	M	Neck (R)
27	28	M	Neck (R)
28	20	M	Neck (L)
29	23	M	Neck (L)
30	34	M	Neck (R)
31	8	M	Neck (L)
32	36	M	Neck (L)
33	30	F	Neck, neck (L)
34	70	F	Axillae, spleen
35	50	M	Neck
36	17	M	Neck (R), mediastinum
37	17	M	Axillae
38	26	F	Neck (L)
39	16	F	Groin (R)
40	22	F	Neck (L)
41	38	F	Neck (R)
42	30	M	Axilla (R)
43	25	M	Groin (L)
44	51	F	Neck (R)

years; in 31 cases the disease began between the ages of 15 and 35 years. Thirty-one cases occurred in men and 13 in women, giving a sex ratio of 2·4 to 1. In all cases where the patient complained of symptoms relevant to the disease the presenting complaint was painless enlargement of lymph nodes, and in 36 of the 44 cases these were situated in the neck. The cases were staged when first seen using the criteria similar to those of Peters and Middlemiss (1958), except that stage II was not subdivided.

Thirty-four cases belonged to stage I, that is to say, had lymph node involvement confined to one group of nodes, six cases belonged to stage II, and four to stage III. Pain was not a feature. Constitutional symptoms were present in two cases in stage I and in one case in stage II. In one patient the spleen was enlarged when she was first seen; the liver was only noted to be enlarged in the terminal stages of the disease. One patient developed a swelling in one buttock which responded to radiotherapy; unfortunately, it was not biopsied. In one case enlarged tonsils were removed; histologically they are said to have shown the typical appearances of benign Hodgkin's disease. Unfortunately, the sections are not now available.

The interval between the onset of symptoms (or signs) and the diagnostic biopsy varied from two weeks to nine years. In six cases the duration of symptoms before biopsy was not recorded, or the lymphadenopathy was an incidental finding in patients attending for other reasons. These cases are recorded as having a biopsy in the first year. In 28 of the remaining 38 cases the interval between the onset of symptoms and biopsy was one year or less, in five cases it was two years, in three cases it was three years, and in the other two cases seven and nine years respectively. These intervals are well substantiated and in the nine-year interval is supported by indisputable radiological evidence. Because of this, the duration of the disease has been measured from the first definite lymph node enlargement; in all cases a positive biopsy has subsequently been obtained from the group of nodes first enlarged.

PATHOLOGY

The affected lymph nodes varied in size between 3 and 50 mm. in diameter, but the majority were between 10 and 30 mm. in diameter. The nodes were discrete and firm, but not hard. Individual nodes were bound together by loose connective tissue; their cut surfaces were smooth, homogeneous, and pale pink or light grey. These appearances are not distinctive and the condition cannot be distinguished from other tumours of lymphoid tissue by naked-eye examination alone.

Partial involvement of affected nodes was surprisingly common, occurring in 16 of 51 biopsies. The uninvolved part of the node was usually small and usually amounted to several follicles surviving near the periphery (Figs. 2, 3, and 4). However, in five biopsies a substantial part of the node (an estimated 20%) retained its normal structure.

Silver-impregnated preparations were available in 41 of 51 biopsies and in our experience were of considerable diagnostic value. They always showed that the normal reticulin pattern in the affected part of the node was completely lost, and incidentally showed up by contrast the frequent presence of small unaffected portions. In the affected part the normal reticulin pattern was replaced by one of three abnormal patterns. In 13 biopsies there was a delicate, sometimes incomplete, meshwork of fine

FIG. 2. *Biopsy 8A from a case of benign Hodgkin's disease showing incomplete involvement of the node. (Note surviving follicle under capsule at bottom left.) (Haematoxylin and eosin × 100.)*

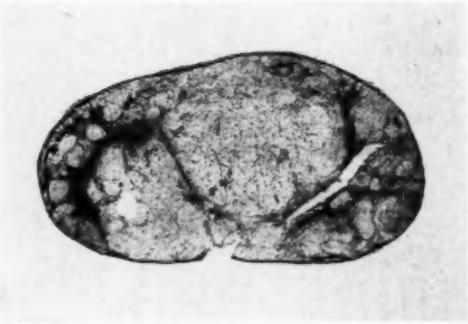

FIG. 3. *Biopsy 14A showing fine reticulin network in the middle of the node and survival of normal reticulin pattern at the two ends. (Silver impregnation × 5·0.)*

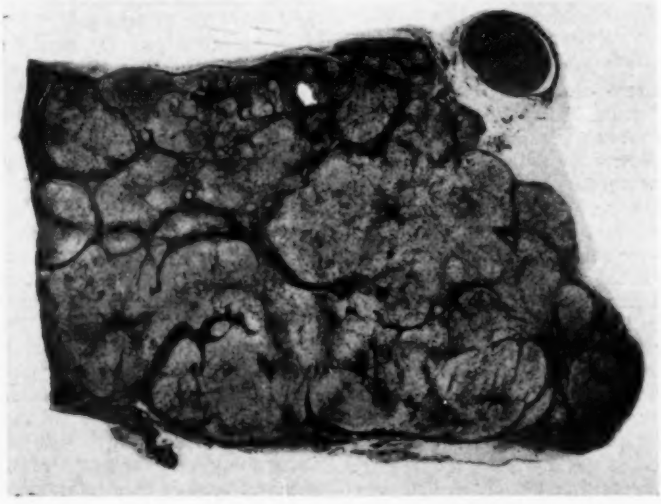

FIG. 4. *Biopsy 12B showing lobulation by reticulin. (Note surviving normal structure in middle by upper border.) (Silver impregnation × 5·2.)*

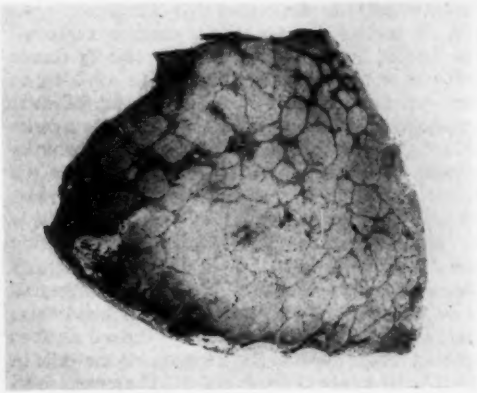

FIG. 5. *Biopsy 43A showing a follicular pattern. (Silver impregnation × 5·2.)*

FIG. 6. *Biopsy 32A from a case of benign Hodgkin's disease, showing the general appearance of massed lymphocytes with a few giant cells. (Haematoxylin and eosin × 100.)*

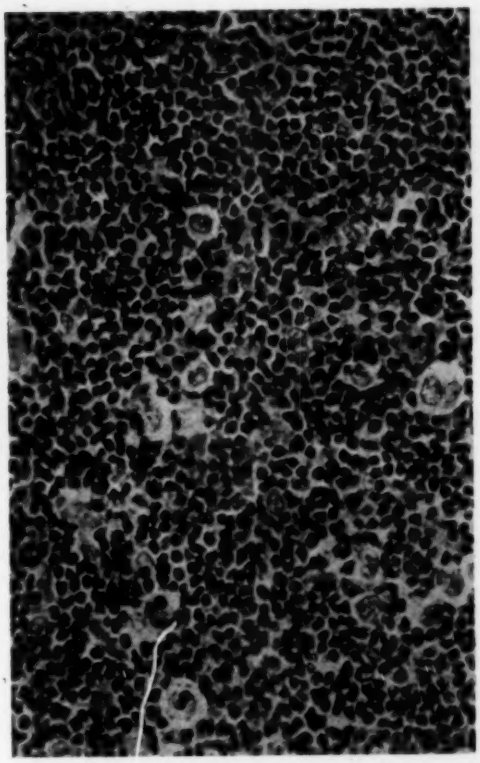

FIG. 7. *Same case as Fig. 6, but at a higher magnification showing lymphocytes and giant cells. (Note mitosis in a Sternberg-Reed cell near bottom margin.) (Haematoxylin and eosin × 380.)*

fibrils, usually less dense than that of a normal node (Fig. 3). In 16 biopsies a similar delicate meshwork was divided into lobules of variable size by denser bands of reticulin fibrils (Fig. 4). In 10 biopsies there was a pattern not unlike that of giant follicular lymphoma. Here the tissue was divided into a series of uniform rounded masses 2 to 4 mm. diameter by reticulin bands with very sparse fibrils within the 'follicles' (Fig. 5). A heavy increase of reticulin was unusual and collagenous bands were seen in only two biopsies. Generally the density of the reticulin was considerably less than in ordinary Hodgkin's disease. In many nodes, including those that appeared to be totally involved, part of the peripheral sinus could still be made out. In ordinary stained sections lymphocytes comprised the majority of the cells in the affected nodes (Figs. 6 and 7). They were morphologically indistinguishable from the small lymphocytes seen in normal nodes and did not show mitotic figures. The reticulum cells, which lay free in the pulp among the lymphocytes, were in the minority. They averaged about 10μ in diameter and had a poorly outlined pink cytoplasm. The nucleus, which occupied most of the cell, had a sharply drawn nuclear membrane, a finely dotted chromatin net and, sometimes, a small dark nucleolus. Mitoses were not commonly seen in these cells and were only present in any number in eight biopsies from seven cases. In one of these cases we have histological evidence of transition to classical Hodgkin's disease, and a further three died with a clinical diagnosis of generalized Hodgkin's disease. In seven biopsies sarcoid-like aggregates of histiocytes were observed. These cells were larger than those just described, with a smaller nucleus and a brightly eosinophilic cytoplasm. Of these seven cases two showed histologically transition to classical Hodgkin's disease and four died, three of generalized Hodgkin's disease.

The relative number of lymphocytes and reticulum cells was found to be of the greatest importance. These were estimated and expressed as a percentage of the total of both types of cell. In 50 biopsies, 80% or more of the cells were lymphocytes and 20% or less reticulum cells. In the remaining biopsy the percentages were 75 and 25 respectively. In 36 biopsies, 90% or more of the background cells were lymphocytes (Fig. 6).

Sternberg-Reed cells were present in all biopsies and were regarded as essential to the diagnosis. They varied in size from 15μ to 45μ in diameter, but the majority lay within the range 15μ to 25μ. Transitions between reticulum cells and Sternberg-Reed cells could be observed fairly readily and for this reason a minimum diameter of 15μ was accepted for the latter (Smetana and Cohen, 1956). Their cytoplasm was poorly outlined, pale pink, and granular, but often vacuolated so that all that could be seen was a halo around the nucleus. The nucleus was big in relation to the size of the cell. Sometimes it appeared single and round, sometimes it was folded or lobed and sometimes there appeared to be two or more round, discrete nuclei. The nuclear membrane was finely drawn, with a few chromatin dots upon it. A striking feature was the presence of one or two large eosinophilic nucleoli measuring from 1μ to 4μ in diameter. Mitoses, both normal and abnormal, were a constant feature of the Sternberg-Reed cells and in only one case were they difficult to find. On average, nearly 3% of all Sternberg-Reed cells were in mitosis (Fig. 7). There was no apparent correlation between the number of mitoses and the liability to transition to classical Hodgkin's disease. The number of Sternberg-Reed cells present varied widely both from node to node and in different parts of the same section. Estimated on a one to four plus system their distribution was: one plus in 14 biopsies, two plus in 22 biopsies, and three plus in 15 biopsies. In no instance were very large numbers of Sternberg-Reed cells seen. In 32 biopsies they were scattered singly through the lymphocytic background, in 18 biopsies they were aggregated to form small loose clusters, and in one biopsy they formed large clusters.

Eosinophils were present in small numbers in seven biopsies, but never in the large numbers observed in some cases of classical Hodgkin's disease. Neutrophil polymorphs were present on one biopsy, and small numbers of plasma cells in four.

Amyloid, staining metachromatically with methyl violet, was present in one biopsy and paramyloid in 21.

Caseation and necrosis were not seen, save in one biopsy where there were a few microscopic areas of necrosis. Capsular invasion was not seen.

DIAGNOSTIC CRITERIA

In our experience the following criteria need to be fulfilled before a diagnosis of benign Hodgkin's disease can be made. The normal architectural pattern of the node in a reticulin preparation must be destroyed in the affected part, and should be replaced by one of the three patterns described above. Sternberg-Reed giant cells must be present. At least 80% of the cells must be small lymphocytes and mitoses in these cells should be absent or rare. Eosinophils should be scanty or absent.

DIFFERENTIAL DIAGNOSIS

Three other malignant lymphomas cause diagnostic

difficulty: lymphosarcoma, giant follicular lymphoma, and classical Hodgkin's disease.

In lymphosarcoma the reticulin pattern shows a loose meshwork like that of one of the patterns in benign Hodgkin's disease. The lymphocytes which form the great majority of the cells show anisocytosis, nuclear variation, and mitotic figures. Small reticulum cells may sometimes be found amongst the lymphocytes but these should not be mistaken for Sternberg-Reed giant cells which do not occur in lymphosarcoma. Capsular invasion is common, a feature which is not seen in benign Hodgkin's disease.

Giant follicular lymphoma can only be confused with the type of benign Hodgkin's disease showing an apparent follicular pattern. Two criteria differentiate these: in giant follicular lymphoma the rounded lesions are lymphoid follicles whereas in benign Hodgkin's disease they are aggregations of lymphocytes without real follicular arrangement. In benign Hodgkin's disease Sternberg-Reed giant cells are present even if scanty: they are absent from giant follicular lymphoma. The differential diagnosis from classical Hodgkin's disease is less clear because the differences are quantitative rather than qualitative. In order to define the differences more closely we compared our 44 cases of benign Hodgkin's disease with 43 cases of classical Hodgkin's disease. In the latter partial involvement of the node was seen only five times. In silver-impregnated preparations the normal architecture was always destroyed and was usually replaced by a tangled network of fibres appreciably denser than in the benign form. The total density of the reticulin was excessive in about two-thirds of cases of classical Hodgkin's disease but in the other third it was not appreciably denser than in the benign form. In seven cases the reticulin showed a fine lobulation like that of one pattern of benign Hodgkin's disease and in another eight there was a similar but much denser lobulation with true collagen. In haemalum-and-eosin-stained sections lymphocytes were always present and they were not morphologically different from those of benign Hodgkin's disease nor did they show mitotic figures. Reticulum cells were always present in much greater numbers than in benign Hodgkin's disease and generally they were larger and more pleomorphic. Mitoses could always be found in them, though they were numerous in only one case. Sarcoid-like aggregates were seen in only five cases. A constant difference was seen in the proportion of reticulum cells to lymphocytes. Whereas in benign Hodgkin's disease reticulum cells did not exceed 20%, they always exceeded 35% in the classical disease (Fig. 9). Sternberg-Reed giant cells were present by definition in both conditions but they were generally more numerous in the classical disease. Using the same grading as we used in benign Hodgkin's disease, we found 1 plus in only five cases, 2 plus in 19, and 3 or 4 plus in the other 19.

In all cases mitoses were observed in the Sternberg-Reed cells and on average they were present in 5%. In 13 cases Sternberg-Reed cells were present in large closely packed groups.

Eosinophils were present in 38 of 43 biopsies and in 29 of these they were present in moderate or large numbers. Neutrophil polymorphs were present in 18 biopsies, in half of them in moderate or large numbers, while plasma cells were present in 21 biopsies, in seven in moderate or large numbers.

Amyloid was not seen but paramyloid was present in 19 biopsies. Caseation was not seen but necrosis was present in nine biopsies and capsular invasion was present in four.

It was originally felt that it might be necessary to create an intermediate group of cases lying midway between benign and classical Hodgkin's disease. Initially, about eight cases fell into this group. Later, after the distinguishing criteria were better established these were re-examined, when they all seemed to fall quite definitely either into the benign or classical group.

COURSE OF BENIGN HODGKIN'S DISEASE

This is summarized in Fig. 1. Characteristically it is slow, and after radiotherapy most patients enjoy a long period of remission during which, although small shotty nodes may still be palpable at the original site, they usually feel quite well. After a varying period many cases relapse. In our series, the longest survival was 26 years from the onset of the disease: another patient who died in the nineteenth year of her illness never received any specific treatment and was only incapacitated by her illness in the last year of her life. The shortest survival was of a patient who died after an illness lasting 15 months. She had widespread lymphadenopathy and a palpable spleen when first seen, so that there is some doubt as to whether the biopsy was truly representative. A total of nine out of our 44 patients have died after periods lasting from 15 months to 19 years, four after more than 10 years. In three of the patients who died there was histological evidence of transition to classical Hodgkin's disease, and this was suggested by the clinical course in another four. We have histological evidence of this transition in two other patients, both of whom are alive and well five and 15 years later respectively (Figs. 8 and 9). The intervals between successive biopsies showing benign and classical Hodgkin's disease in the cases

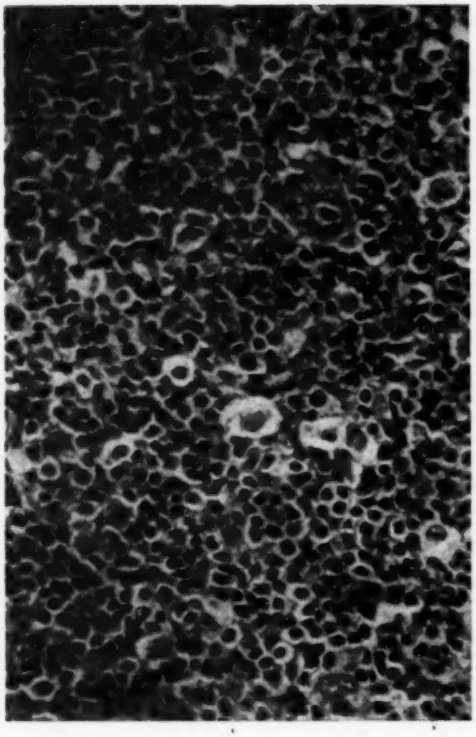

FIG. 8. *Biopsy 3A, the original biopsy from a case of benign Hodgkin's disease.*
(*Haematoxylin and eosin* × *380.*)

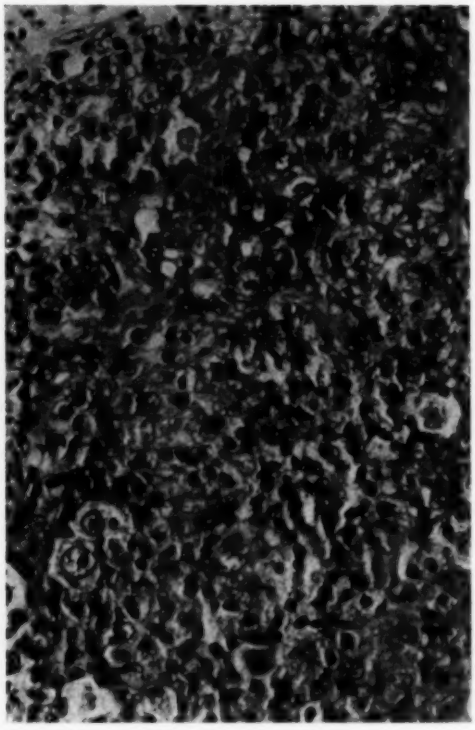

FIG. 9. *Biopsy 3B, six years after biopsy 3A, now showing classical Hodgkin's disease. The majority of the cells are now reticulum cells and lymphocytes are scanty.*

in which transition occurred varied from four to 13 years.

In the other two fatal cases post-mortem examination revealed generalized amyloidosis, and in both death was due to uraemia consequent upon renal vein thrombosis. In one case the benign Hodgkin's disease was confined to a single group of nodes in the neck. In the other there was no trace of Hodgkin's disease and, although the clinical course was punctuated by frequent recurrences, three biopsies taken over a period of nine years all showed benign Hodgkin's disease. Intercurrent illnesses were not prominent in our series and none of the deaths could be ascribed to them apart from the above cases of amyloid which were considered to be secondary to benign Hodgkin's disease.

Treatment was by local excision, radiotherapy, or nitrogen mustard, either singly or in combination. Local excision was the only form of initial treatment in 15 cases and in only one of these was a radical dissection attempted. In 10 of these the disease recurred at the same site after intervals varying from one to 13 years, and in one case residual enlarged nodes have remained unchanged. The remainder are free from recurrence, one, four, four, and 20 years later respectively.

Thirty-four patients received one or more courses of radiotherapy; another patient also received radiotherapy but only after transition to classical Hodgkin's disease had occurred. Details of the dose and method are not generally available. A single course of treatment was followed, in 24 patients, by a remission lasting from three to 10 years; only five of these have had recurrences. In five patients several courses of treatment were required before remissions lasting from five to 22 years were induced.

In four cases the disease was never satisfactorily controlled and ran a relentless course with death after illness lasting from 15 months to 12 years. The remaining patient has been observed for less than one year since radiotherapy; he is free from recurrence.

Chemotherapy was only employed as the initial treatment in two cases.

The period of follow-up has varied from a minimum of four years in all cases to a maximum of 26 years. Of 41 patients followed for five years, three died, giving a crude five-year survival rate of 93%. Of 19 patients followed for 10 years, five died, giving a crude 10-year survival rate of 74%. A better estimate of the survival rate is given by the life table (Table II); calculated by this method, the five-year survival rate is 93% (standard error=3·8%) and the 10-year survival rate is 85% (standard error=6·5%).

DISCUSSION

INCIDENCE OF BENIGN HODGKIN'S DISEASE Because of the way in which the present series was collected, it was impossible to determine from our figures the incidence of benign Hodgkin's disease. Of 2,127 cases of tumours of lymphoid tissue reported in the literature (Jackson and Parker, 1947; Lumb, 1954; Symmers, 1958), 925 (44%) were examples of Hodgkin's disease. Of these 113 were cases of benign Hodgkin's disease. The incidence of benign Hodgkin's disease is thus approximately 12% of all cases of Hodgkin's disease and 5% of all tumours of lymphoid tissue. Wright (1960) found that of 339 cases of Hodgkin's disease 26 (8%) were examples of the benign form.

CLINICAL FEATURES The published series of benign Hodgkin's disease are summarized in Table III. The

TABLE II

LIFE TABLE OF SURVIVAL RATES IN BENIGN HODGKIN'S DISEASE

Time in Years	*No. of Cases Alive at Beginning of Each Year*	*No. of Cases Alive but Falling out of Follow-up*	*No. of Cases Dying in Given Year*	*No. at Risk in Given Year*	*Probability of Dying in Given Year*	*Probability of Surviving through Given Year*	*Survival Rate (%)*
1	44	0	0	44	0	1	100
2	44	0	1	44	0·02273	0·97727	97·7
3	43	0	0	43	0	1	97·7
4	43	0	1	43	0·02326	0·97674	95·5
5	42	3	1	40·5	0·02469	0·07531	93·1
6	38	8	0	34	0	1	93·1
7	30	4	0	28	0	1	93·1
8	26	3	1	24·5	0·04082	0·95918	89·3
9	22	2	1	21	0·04762	0·95238	85·0
10	19	5	0	16·5	0	1	85·0

TABLE III

SEX AND AGE INCIDENCE OF BENIGN HODGKIN'S DISEASE

Author(s)		*Decade*						
		1st	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th*	*8th*
Jackson and Parker (1947)[1]	M 30 F 11	2	6	9	7	5	5	6	1
Jelliffe and Thomson (1955)[1]	M 6 F 2	—	2	3	1	—	2	—	—
Wright (1956)[2]	M 8 F 2	—	—	3	3	1	3	—	—
Lumb and Newton (1957)[2]	M 33 F 3	—	6	15	5	3	4	3	—
Symmers (1958)[1]	M 44 F 7	1	8	19	12	5	3	3	—
Wright (1960)[2]	M 21 F 3	—	2	11	2	7	2	—	—
Totals	M 142 F 28	3	24	60	30	21	19	12	1
Present series[3]	M 31 F 13	3	11	15	9	1	4	—	1

[1]Age at onset. [2]Age at diagnosis.
[3]Lumb and Newton decades are from 0 to 10 years, 10 to 20 years, etc., whereas in the other series they are from 0 to 9 years, 10 to 19 years, etc.

combined ratio men to women is 15·1 to 1 compared with that in the present series of 2·4 to 1, but some of the published series may be biased because of the authors' connexions with the Armed Forces. Our present figures correspond closely with those of Jackson and Parker, and with those for Hodgkin's as a whole (1·8 to 1 in favour of males; collected series quoted by Hoster, Dratman, Craver, and Rolnick, 1948). Benign Hodgkin's disease is rare under the age of 10 years and has not been reported under the age of 5 years. It is most common between the ages of 15 and 35 years.

SITE OF ONSET AND CLINICAL STAGE Approximately three-quarters of all the cases reported in the literature initially involved only a single group of nodes, generally in the neck; our findings are similar. The existence of benign Hodgkin's disease outside lymph nodes is still uncertain. Jackson and Parker (1947), Marshall (1956) and Lumb and Newton (1957), all noted occasional involvement of the spleen and bone marrow clinically and Symmers (1958) stated that leukaemia could occur. Wright (1960) observed at necropsy infiltration of the liver and spleen by collections of lymphocytes and Sternberg-Reed cells, although elsewhere in the body the picture was that of classical Hodgkin's disease. In the present series the disease appeared to have involved bone marrow, spleen, spine, breast, and soft tissues of the buttock. In none of these was the involvement confirmed histologically, so that it is uncertain whether or not transition to the classical form had occurred; in some cases it appeared likely that it had. Cases showing splenomegaly or other evidence of spread beyond lymph nodes usually do badly.

Jackson and Parker (1947) state that constitutional symptoms do not occur. Three of our patients complained of weight loss and fever; in two of these the disease was apparently confined to a single group of nodes. One patient remained well for eight years following radiotherapy before transition to classical Hodgkin's disease occurred, and one remained well for four years following her initial course of treatment, before developing enlarged lymph nodes in the abdomen. The remaining patient ran a progressive course, dying three years after the onset of the disease. Four patients have been, or are, pregnant. Hartvigsen (1955) and Southam, Diamond, and Craver (1956) found that in patients with Hodgkin's disease who became pregnant the survival time was significantly reduced. Patients did badly particularly if their pregnancy coincided with an active phase of the disease. All the pregnancies in our series occurred during quiescent periods of the disease and, so far, no ill effects have been observed.

Two of our patients died from amyloidosis secondary to benign Hodgkin's disease. Castleman (1949) reports a similar case and Jackson and Parker (1947) report a case in which a transition from benign to classical Hodgkin's disease had occurred. The association of amyloidosis with classical Hodgkin's disease is fairly well recognized, although the number of cases in the literature is only 63 (Dawson, 1960).

HISTOPATHOLOGICAL FEATURES While broadly following the description of Jackson and Parker (1947), subsequent descriptions (Harrison, 1952; Lumb, 1954; Smetana and Cohen, 1956; Wright, 1956; Lumb and Newton, 1957; Symmers, 1958; Pitcock, Bauer, and McGavran, 1959; Wright, 1960) have differed in detail and emphasis, and new facts have emerged.

Partial involvement of affected lymph nodes was first described by Jackson and Parker and stressed by Harrison, although Wright and Symmers believe it to be infrequent. The present series has shown it to be surprisingly frequent and the retention of a few surviving follicles to one side of the node in no way militates against the diagnosis.

The reticulin pattern varies widely. Harrison first drew attention to the reticulin and collagenous lobulation, an observation since confirmed by Lumb and Wright as well as by the present findings. Rappaport, Winter, and Hicks (1956) described a follicular lymphoma of Hodgkin's type of which they wrote 'were the background not follicular or nodular, one would not hesitate to classify most of these as Hodgkin's paragranuloma'. These authors showed that the presence of a follicular pattern in any type of tumour of lymphoid tissue was associated with an improved diagnosis. In our series, a fine network with or without superadded lobular and follicular pattern was observed. There is thus no single characteristic reticulin pattern in this disease, although a gross increase in the amount of reticulin is against the diagnosis and in favour of classical Hodgkin's disease.

All authors stress that the bulk of the node is composed of small mature lymphocytes, which cannot be distinguished morphologically from those seen in normal nodes. Mitotic figures are not seen in these cells. The importance of the number of lymphocytes was first realized by Rosenthal (1936) and was the basis of his attempt to correlate histological appearances and prognosis; this has recently been emphasized by Pitcock *et al.* (1959), who state that 'the length of survival correlated at the 5% level of probability with the amount of lymphoid background'. As a result of the present study, it is now possible to lay down a lower limit of 75% for the lymphocytes in the background. In most nodes they will total 90% or more of the background cells.

Jackson and Parker stated that reticulum cells were present in varying numbers. Harrison commented on their virtual absence and Lumb thought that an increase in proliferating reticulum cells was associated with a liability to malignant change. The present study has shown that the number of reticulum cells is inversely proportional to the number of lymphocytes and is usually less than 10% of the total background cells. A percentage greater than 25%, to our mind, precludes a diagnosis of benign Hodgkin's disease. The presence of mitotic figures in these cells has been shown to be of bad prognostic significance, and indeed we pay more attention to the number of reticulum cells and the presence of mitoses in them than to the lymphocytes. Sarcoid-like lesions have been reported in classical Hodgkin's disease by Smetana and Cohen (1956) and in benign Hodgkin's disease by Jackson and Parker (1947). These lesions were observed in seven biopsies in the present series and appear to be associated with a poor prognosis, although this was not the experience of Smetana and Cohen. Sternberg-Reed cells are considered by all authors to be essential for the diagnosis of benign Hodgkin's disease, although Wright (1960) calls them 'reticulum cells'. They vary widely in number and are indistinguishable from those found in other forms of Hodgkin's disease. Lumb makes the reservation that giant forms are not common and Symmers thinks that the presence of bizarre forms forebodes transition to the classical form. On the whole they are present in small or moderate numbers only and are characteristically scattered singly or in loose clusters through the predominantly lymphocytic background. Harrison first drew attention to the constancy of mitotic figures in these cells, and nearly 3% of all Sternberg-Reed cells observed in the present series were in mitosis. Both normal and abnormal mitotic figures were observed. There is no direct correlation between mitotic activity and prognosis.

Jackson and Parker observed small numbers of eosinophils and plasma cells, a feature confirmed by later authors and ourselves. Caseation, necrosis, and capsular invasion do not occur. Paramyloid was a frequent finding in the present study.

Transition from benign to classical or sarcomatous Hodgkin's disease has been observed by most authors (Jackson and Parker, 1947; Custer and Bernhard, 1948; Lumb and Newton, 1957; Symmers, 1958; Wright, 1960), although the frequency with which this occurred has varied from series to series. Jackson and Parker estimated the frequency at about 20%, and 10 of Lumb and Newton's 36 cases underwent this change. We were able to confirm this transition histologically in five cases, and its occurrence was suggested by the clinical course in a further four. Although Smetana and Cohen (1956) felt that this transition was more apparent than real and was due at least in part to misdiagnosis of the original biopsy, it now seems established as a definite occurrence in perhaps 25% of cases, and as the major cause of death. We believe that this change represents a phase in the natural history of the disease and is evidence for a close aetiological relationship between the two forms. It is impossible with the present state of knowledge to predict which cases will undergo this transition, but in our experience the presence of mitotic figures in reticulum cells and sarcoid-like areas are associated with a relatively poor prognosis. In most cases transition occurs between the fifth and fifteenth year of the disease, although Jackson and Parker (1947) describe a case in which it occurred in the twenty-fourth year.

SURVIVAL RATES IN BENIGN HODGKIN'S DISEASE The comparative survival rates reported in the literature for the various types of Hodgkin's disease are given in Table IV. There seems some doubt as to the diagnosis in Hall and Olson's (1956) small series of five cases so these are excluded. From these figures it is readily apparent that these results are much better than the figures generally reported for Hodgkin's disease.

TABLE IV

FIVE-YEAR SURVIVAL RATES AND HISTOLOGICAL CLASSIFICATION IN HODGKIN'S DISEASE

Author(s)	*Classification*	*No. of Cases*	*No. of Cases Surviving at Five-year Follow-up*
Jackson and Parker (1947)[1]	Paragranuloma	28	15 (55%)
	Granuloma	136	18 (13%)
	Sarcoma	32	Nil
Peters (1950)[2]	Early	20	15 (75%)
	Classical	49	25 (57%)
	Late	24	11 (50%)
	Atypical	20	5 (25%)
Jelliffe and Thomson (1955)[1]	Grade I	5	5 (100%)
	Grade II	156	45 (29%)
	Grade III	10	Nil
Smetana and Cohen (1956)[2]	Paragranuloma	35	(77% net)
	Granuloma	308	(28% net)
	Sarcoma	5	Nil
Lumb and Newton (1957)[1]	Reticular lymphoma	29	27 (93%)
	Hodgkin's disease	194	67 (35%)
Wright (1960)[2]	Paragranuloma	19	18 (95%)
	Ordinary Hodgkin's	157	43 (27%)
	Hodgkin's sarcoma	10	1 (10%)

[1]Survival period calculated from onset of symptoms
[2]Survival period calculated from biopsy or treatment

This difference becomes much more significant if the 10-year survival rates of benign and classical Hodgkin's disease are compared. Twenty-one of the cases reported by Jackson and Parker (1947) were followed for 10 years; 10 (48%) were alive at the end of this time. Smetana and Cohen (1956) reported a 55% 10-year survival rate. Wright (1956) followed five cases for 10 years with no deaths; but in a later series (1960) of 11 cases followed for the same period three died. Fourteen of 19 of our cases followed for 10 years survived this period. These results are very different from those quoted for Hodgkin's disease as a whole (see Table V) which

TABLE V

10-YEAR SURVIVAL IN HODGKIN'S DISEASE

Author(s)	*No. of Cases at 10-year Follow-up*
Unselected for histological type	
Uddströmer (1934)	494 (Nil)
Slaughter and Craver (1942)	265 (3%)
Merner and Stenstrom (1947)	185 (8%)
Healy, Amory, and Friedman (1955)	216 (6%)
Peters and Middlemiss (1958)	139 (29%)
Excluding cases of benign Hodgkin's disease	
Jackson and Parker (1947)	136 (1%)
Lumb (1954)	123 (2%)

vary between nil and 29%. In the series of Jackson and Parker, and of Lumb, where cases of benign Hodgkin's disease are excluded, only one of 136 and two of 123 cases respectively survived 10 years.

The results of Peters and Middlemiss (1958) are much better than average and must be attributed to a better technique of treatment. It is their practice to irradiate prophylactically the lymph node regions adjacent to those actually affected. In the present series treatment was undertaken at many centres so that the results cannot be a reflection of a single technical advance in radiotherapy.

It is, however, difficult in a disease with such a slow remittent course to assess accurately the effect of treatment in the individual case. The striking difference in the recurrence rates after surgery alone, and after radiotherapy, observed by both Wright and ourselves suggests that radiotherapy does in fact influence the course of the disease. For this reason we advise biopsy-excision followed by radiotherapy.

Peters (1950) first showed a correlation in Hodgkin's disease between the extent of the disease when the patient was first seen and the survival rate. Because of the frequency with which benign Hodgkin's disease is initially localized to a single group of nodes, it has been suggested that the improved prognosis is merely a reflection of the localization of the disease. If the present series is combined with that of Wright (1956) and the others listed in Table IV, a total of 151 cases is obtained; of these 127 survived five years. This gives a crude five-year survival rate of 84%. Two hundred and fifty-nine cases of Hodgkin's disease, clinically localized to a single lymphatic region, mostly unselected for histological type, are listed in Table VI. (Lumb and Newton exclude cases of what they

TABLE VI

FIVE-YEAR SURVIVAL IN HODGKIN'S DISEASE ACCORDING TO CLINICAL STAGE

Author(s)	*Clinical Stage*	*No. of Cases*	*Five-year Survival: No. of Cases*
Jelliffe and Thomson (1955)	I	32	19 (59%)
	II	47	28 (60%)
	III	92	3 (3%)
Sellers (1957)	I	100	57 (57%)
	II	141	49 (35%)
	III	174	20 (12%)
Lumb and Newton (1957)	I	67	35 (52%)
	II	67	27 (40%)
	III	60	5 (7%)
Peters and Middlemiss (1958)	I	60	43 (71%)
	II	70	39 (56%)
	III	91	14 (15%)

call 'reticular lymphoma'.) Of these, 154 survived five years, giving a crude five-year survival rate of 59%. There is thus a considerable difference in prognosis between cases of Hodgkin's disease localized to one site and cases of benign Hodgkin's disease, irrespective of the extent of the disease when first seen. These arguments in no way invalidate Peters' conclusions and it appears to be an established fact that the prognosis in classical Hodgkin's disease is much better when the condition is localized to a single group lymphatic region.

As far as the individual patient is concerned, it is extremely difficult to state dogmatically whether a cure has been effected in benign Hodgkin's disease. Certainly recurrences and death after a quiescent period of 10 years or longer are not uncommon. Jackson and Parker (1947) report a patient who died in the twenty-seventh year of his illness; following his initial course of treatment he had remained well for 24 years. The same authors also report a patient surviving 35 years with recurrences in the seventh and twenty-seventh years. We believe that progression is ultimately inevitable in the absence of treatment but that with energetic radiotherapy a permanent cure is possible.

This work was made possible by the many colleagues who sent us cases and helped us so generously with the follow-up; to them we are deeply grateful. We should also like to thank Professor I. Doniach for much helpful advice.

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Tumours of salivary tissue

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SYNOPSIS A clinical study of 401 cases of tumour of salivary tissue has been made and the results reported, together with a review of the literature. The histological variations of such tumours are classified and discussed.

Since the first attempt by Bérard (1841) tumours of salivary tissue have not been satisfactorily classified either clinically or pathologically. Attention has been focused mainly on the so-called 'mixed tumours', and other varieties have often been neglected. The classification used throughout this paper is a modification of that of Willis (1953) and the classification together with the number of cases is shown in Table I.

TABLE I

CLASSIFICATION OF SALIVARY TUMOURS

Type		*No.*
I	Simple adenoma	—
II	Pleomorphic adenoma	315 (78·5%)
III	Carcinoma	64 (16%)
IV	Adenolymphoma	16 (4%)
V	Miscellaneous	6 (1·5%)
Total		401 (100%)

TYPE 1: SIMPLE ADENOMA

The term 'adenoma' was introduced by Lebert and Broca (1850) for benign tumours of the parotid gland. It is generally agreed that there is no sharp line of demarcation on structural grounds between the simple adenoma and the pleomorphic adenoma. According to Willis (1953), pure adenomas are slowly growing, encapsulated, lobulated, epithelial tumours. They may, however, be only a variant of the whole group, namely, the most slowly growing and innocuous variety with a uniformly differentiated structure. Some 40 cases have been described in the European literature; the first in the British literature was by Dunlop (1879) in a 36-year-old woman at the Glasgow Royal Infirmary.

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No case of simple adenoma was observed in this series as the distinction between simple adenoma and pleomorphic adenoma is so artificial that the first two types can be grouped together. With this in mind, only the last four types of salivary tumours will be considered.

TYPE 2: PLEOMORPHIC ADENOMA

Pleomorphic adenoma is a bizarre lesion which accounts for the vast majority of all salivary tumours. There are in this series 315 examples, constituting 78·5% of the total. Although the first published reports of such tumours are generally credited to C. G. Siebold (1793) and J. B. Siebold (1797) little of morphological interest has been added since 1853, when it was recognized as a clinical entity by Paget, and since Theodor Billroth wrote his classical thesis in 1859. The term 'mixed tumour' dates from Minssen's review in 1874. Many authors have reviewed the literature since Wood (1904), notable reviews being by Wilson and Willis (1912), Heineke (1913), Kennon (1921), and McFarland on numerous occasions (1926, 1933, 1936, 1942, 1943).

Fick (1909) and Patey (1930) rightly suggest that the confusion over terminology is due to the dispute as to its aetiology. For this reason such hyphenated monstrosities as chondro-myxo-haemato-endotheliosarcoma have been used. Most of the early German pathologists considered them of connective tissue origin and any epithelial structures as vestiges of the gland in which they occurred. In 1880 Wartmann expressed the opinion that they were derived from lymphatic endothelium. Volkmann (1895) thought that he had proved the endothelial origin as did Martini (1907), von Hansemann (1910), and others. This theory was adversely criticised by Hinsberg (1899) who, with Cunéo and Veau (1900), Wood (1904), Chevassu (1910), and Ssobolew (1912), suggested a branchiogenic origin and so explained the

TABLE II

SITES OF 315 PLEOMORPHIC SALIVARY ADENOMATA COMPARED WITH SERIES OF PREVIOUS WORKERS

Author(s)		*Parotid*	*Submandibular*	*Sublingual*	*Palate*	*Lip*	*Miscellaneous*	*Total*
Present series	R.	142	8	0				
	L.	123	11	1	14	9	7	315
	Total	265	19	1	14	9	7	315
Wood (1904)		35	13	0	3	4	4	59
Schreiner and Mattick (1929)		45	7	0	1	9	4	66
Patey (1930)		38	6	1	5	1	3	54
Zymbal (1933)		55	2	0	0	1	0	58
Harvey *et al.* (1938)		230	21	2	6	9	7	275
Willis (1953)		35	6	1	5	1	0	48
Ross (1955)		84	9	0	3	0	2	98

presence of 'cartilage' and 'bone'. Other suggested theories at this time included that of Forgue and Massabuau (1908) that they should be classified as embryomata along with testicular tumours. Teratoma of the neck have been reported by McGregor and Workman (1906) and others, but these tumours are quite different. Another theory which did not get many supporters was that of Krompecher (1908) who regarded them as basal cell tumours. Wilms (1899), Marchand (1910), and others regarded them as tumours in which the epithelium plays the most important part. Fraser (1918) with his work on dogs believed he had experimental proof that they arose from the ducts of adult glands. He is supported by Fry (1927). McFarland (1926), supported by Kux (1931) and Hemplemann and Womack (1942), suggests that the origin is probably sequestration of embryonal cells. Many agree on the purely epithelial nature of the tumours (Fick, 1909; Böttner, 1921; Fry, 1927; Patey, 1930; Zymbal, 1933; Harvey, Dawson, and Innes, 1938; Muir, 1941) and others who have argued for an essentially epithelial origin have accepted without question the belief that they contain 'cartilage' and have devised various explanations of its origin. Harvey *et al.* (1938), however, suggest that it is not cartilage but pseudo-cartilage or a myxochondroid substance developed from epithelium and stroma by degeneration. The epithelial cells which are isolated in a retraction space in the acellular homogenous mucoid matrix give all the appearance of cartilage cells within their capsule. Fry (1927) suggests that a similar appearance is given by a ring of deeply staining mucin round isolated cells.

By far the majority of pleomorphic adenomata arise in the parotid gland, less commonly the submandibular, and rarely in the sublingual gland. They are, however, also found in the palate and various parts of the oral-facial region such as the lips, nose, pharynx, or lacrimal glands, arising probably from serous or mucous glands.

The site distribution reported by several workers is contrasted with the present series in Table II. It will be seen that the parotid gland accounts for just over 80% of such tumours. Other series vary from 70% (Sirsat, 1953) to 98% (Montella and Fontana, 1956). Of these 265 tumours occurring in the parotid gland, 142 cases (53·6%) occurred on the right side; although not statistically proved, this slight preference for the right side agrees with the findings of Wilson and Willis (1912) and Benedict and Meigs (1930) while McFarland (1943) states that the left is more frequently involved. From these findings, however, there seems to be no preference for any side.

Submaxillary tumours have been specially considered by Chevassu (1910) and Dockerty and Mayo (1942) while S. Paget (1886), Eggers (1928), D'Aunoy (1930), Davis (1935), and Stobie (1935) discussed palatal tumours. Lacrimal gland tumours were considered by Verhoeff (1905) and those of the lip by J. Paget (1853) and more recently by Bernier (1946).

Most authors consider that the commonest age for the first appearance of the tumour is the third and fifth decade. McFarland (1926) considered 66% occurred before the age of 40 years and 90% before the age of 50 years. In this series almost 60% occurred before the age of 40 years and 90% before 60 years of age. Table III shows the distribution of

TABLE III

DECADE DISTRIBUTION OF AGES AT FIRST APPEARANCE OF THE TUMOUR

Decade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Total*
Male	1	17	20	34	21	15	9	5	1	0	123
Female	3	21	41	46	40	26	12	2	1	0	192
Total	4	38	61	80	61	41	21	7	2	0	315

the age at the first appearance of the tumour in this series by decades. No age is exempt and it is not unusual in childhood. According to Byars, Ackerman, and Peacock (1957) 5% occur before the age of 18 years. Occasionally the condition is first encountered in infancy; Pailler (1903) reported a case of a mixed tumour in a child of 11 months, and

Wood (1904) in an infant of 7 months. Examples reported occurring in the first decade include those of Chevassu (1910) and Zymbal (1933) 9 years and Fry (1927) at 10 years, and Schilling (1921) reports a tumour occurring in a man aged 41 that had been present since he was 1 year old. McFarland (1942) out of 380 cases found eight occurring in the first decade. Of the four occurring in the first decade in the present series, the earliest age of first appearance of the tumour was 3 years, it being treated conservatively until the patient was 42 years of age. The remaining three tumours all appeared at 9 years of age with surgery being undertaken three, four, and five years later respectively. Most tumours exhibited a slow, intermittent growth and on an average six years elapsed from the time of first appearance until the initial operation. Table IV shows the decade

TABLE IV

DECADE DISTRIBUTION OF AGES AT TIME OF FIRST OPERATION

Decade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Total*
Male	0	10	17	27	28	18	13	9	0	1	123
Female	0	9	34	35	41	49	14	9	1	0	192
Total	0	19	51	62	69	67	27	18	1	1	315

distribution of the age at the time of the first operation in this series. The longest duration before the patient was subjected to surgery was 39 years, but Street (1913) and Ross (1955) report cases with intervals of 40 and 60 years respectively before the patient received surgery.

Although in recent years most reported series show a predominance of females (Table V), most

TABLE V

SEX DISTRIBUTION OF PLEOMORPHIC ADENOMATA IN PRESENT SERIES COMPARED WITH FIGURES GIVEN BY PREVIOUS WORKERS

Authors	*Male*	*Female*
Wood (1904)	25	21
Chevassu (1910)	30	27
Wilson and Willis (1912)	30	26
Böttner (1921)	10	15
Fry (1927)	11	14
Ahlbom (1935)	106	148
McFarland (1942)	184	212
Willis (1953)	16	34
Fitzgibbon (1953)	19	23
Present series	123	192

earlier workers stated that the sexes showed little difference in their liability to pleomorphic adenomata of salivary tissue.

The largest tumour on record seems to be that reported by Cotterill (1907) which is said to have been twice the size of a man's head and to have weighed approximately 26 lb. (Fig. 1). As these

FIG. 1. *Photograph of Cotterill's case.* (*By permission of the publishers, W. Green & Son, Ltd., Edinburgh.*)

tumours have in the past received only a passing mention, details were often scanty and the weight, when it was mentioned, was not necessarily accurate. Witness Keen's (1904) case in which the tumour was said to be 7 lb., the excised tumour weighing 4¾ lb. at operation but an allowance of 2 lb. was made for the fluid lost when the tumour was 'burst' a year before the operation. Amongst the largest surgically treated and confirmed histologically as mixed are those shown in Table VI. The patient of Short and Pullar (1956) is included in this series (Case 221) as she had been treated at the Glasgow Royal Infirmary between 1935 and 1956.

Opinions vary as to the rate of recurrence; for example, Wood (1904) 30%, Benedict and Meigs (1930) 42·5%, Stein and Geschickter (1934) 20%, and McFarland (1942) 25%. Recurrences are seen more commonly in the parotid tumours, possibly because of anatomical relations (Patey, 1940). In this series the recurrence rate was 29·5%, and the details of these recurrences together with their sites are seen in Table VII. The average interval between the original operation and subsequent excision in this series was four years.

TABLE VI

OTHER GIANT PAROTID TUMOURS CLASSIFIED HISTOLOGICALLY AS 'MIXED'

Authors	*Weight (kg.)*	*Size*	*Sex and Age (yr.) at Onset*	*Duration (yr.)*
Spence (1863)	3·1	9½ × 8½ × 7½ in.	F. 18	16
Rouville and Martin (1904)	2·95	—	F. 14	30
Ahlbom (1935)	2·25	—	F. 30	17
Frazell (1954)	1·27	22 cm. diam.	F. 38	6
Cade (1954)	1·99	19 × 15 × 13 cm.	M. 54	18
Short and Pellar (1956)	2·8	30 cm. diam.	F. 33	16

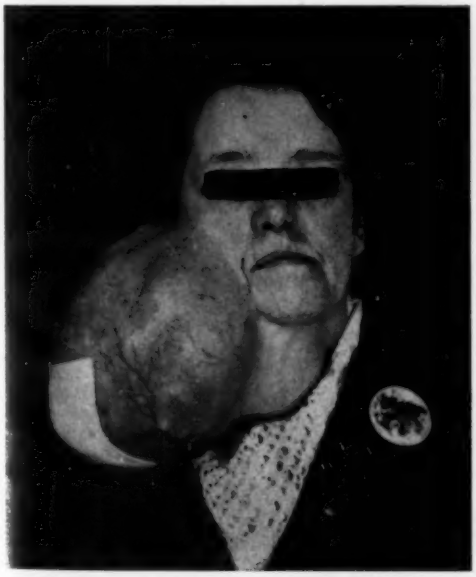
TABLE VII

RECURRENCE RATE OF PLEOMORPHIC ADENOMATA AT SITES OF ORIGIN

	Parotid	*Submandibular*	*Sublingual*	*Palate*	*Lips*	*Miscellaneous*	*Total*
Recurrences	78	7	1	2	1	4	93
Non-recurrences	187	12	0	12	8	3	222
Total	265	19	1	14	9	7	315

Although no reference could be found of any previous report of pleomorphic adenomata affecting more than one member of a family, a familial incidence was found in this series on three occasions (Cameron, 1959). Likewise a highly significant percentage of the patients with pleomorphic adenomata in this series belong to blood group A (Cameron, 1958).

Reference has already been made to the varied structure of pleomorphic adenomata and now only the commonest variations which were encountered will be shown. The findings accord with the descriptions of the earliest workers, in particular of Zymbal (1933) and of Willis (1953), the former giving by far the best account of the microscopical structure of salivary tumours. In some tumours, many or all the possible variations of structure may be found in juxtaposition with all gradations between them; while in others, one or another structural variant predominates.



IG. 2. *Case recorded by Short and Pullar in 1956 Case 221 in this series).*

The atypical glandular structure is seen in a great variety of forms. They may be acinar or ductular in type, cystic or papillary, and they may contain mucoid or hyaline material. Predominance of the goblet-cell epithelium is sometimes seen, as in the cases of Schilling (1921), Lepp (1939), De and Tribedi (1939), Škorpil (1940), Linell (1948), and Cooray, Tennekoon, Kanakaratne, and Attygalle (1950). On occasion it is associated with squamous-cell epithelium and some workers believe that such tumours form a distinct group, namely the 'muco-epidermoid' tumours, and state that they account for approximately 50% of salivary tumours. According to Du Plessis (1957), in 28% of normal parotid glands, sebaceous glands may arise from the parotid ducts, thus explaining the site of origin of such muco-epidermoid tumours.

The classical ductular type is seen in Fig. 3 while a relatively common pattern (Fig. 4) is that in which the glandular tissue merges into the mucinous matrix. This finding is well described by Zymbal (1933), and is shown by epithelial sprouting from the glandular formations. This fraying out of the epithelium is a common way of development of the so-called 'mucoid tissue'. A feature not uncommonly encountered is the presence of extensive areas of squamoid change (Fig. 5).

Many tumours contain a fairly solid glandular

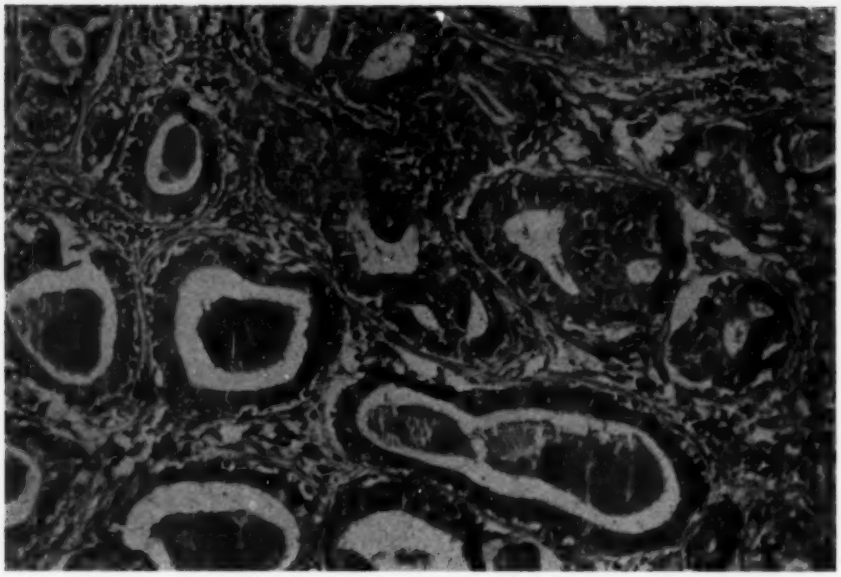


FIG. 3. *Photomicrograph showing a glandular type of pleomorphic adenoma. Haematoxylin and eosin × 200.*

FIG. 4. *The glandular tissue is merging into the mucoid matrix. Haematoxylin and eosin × 770.*

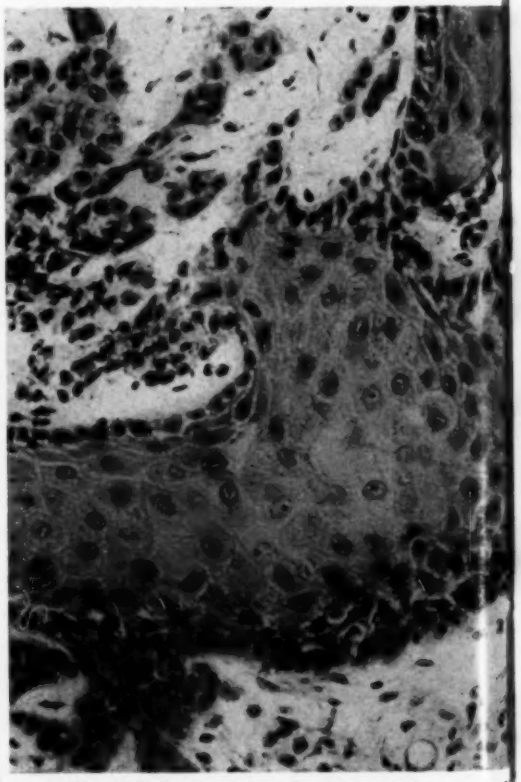


FIG. 5. *A high-power view showing extensive area of squamoid change. Haematoxylin and eosin ×800.*

epithelium. The case illustrated in Fig. 6 was the only one in the series which bore any resemblance to simple adenoma. In solid epithelial tumours of this type the glandular structures may be arranged in tubular or cylindromatous fashion (Fig. 7) or acinar formation (Fig. 8). In large epithelial masses rounded or irregular spaces may appear, chiefly by secretory products of the cells collecting. Sometimes the secretory spaces assume a characteristic structure, cribriform in type (Fig. 9), which resembles that seen in mammary tumours (Willis, 1953).

The proportions of the epithelial elements to the matrix is very variable and a case previously described as 'myxo-chondroid' where the matrix reveals its looseness with stellate cells is seen in Fig. 10. A frequent finding in many tumours is the presence of areas of 'pseudo-cartilage' (Fig. 11) and in rare cases even bone (Allen, 1940; Yates and Paget, 1952). It is my belief that cartilage and even bone do occur in pleomorphic adenomata on occasion, although probably not as frequently as the pseudo-cartilage so commonly talked about.

TYPE 3: CARCINOMA

As Fry (1927) suggests, there are varying degrees of malignancy and there is again no sharp line of demarcation between an unusually active pleomorphic adenoma and frank carcinoma. The incidence, however, is higher than is generally appreciated, reports varying from 50% to 15%, although usually between 17% and 25%; in this series it accounts for 16% of the total of salivary tumours (Table VIII). On an average, according to Foote and Frazell (1954), the age incidence is 10 years older than in pleomorphic adenomata, while Quattlebaum, Dockerty, and Mayo (1946) give the average age as 49·8 years. Dick (1954), however, reported a case of a small-cell carcinoma arising in the left cheek of a 2-day-old boy. In this series the commonest age of the first appearance of the tumour is between the fifth and eighth decades (Table IX). In view of the more rapid increase in size and the associated symptoms of pain and/or paralysis, there is a shorter interval before the patient receives treatment, in this series two to three years on an average (Tables IX and X).

TABLE VIII

PERCENTAGE INCIDENCE OF SALIVARY CARCINOMATA

Nasse (1892)	11
Wood (1904)	25
Kennon (1921)	22
Benedict and Meigs (1930)	42
Stein and Geschickter (1934)	15
Slaughter *et al.* (1953)	26
Ross (1955)	22·8
Present series	16

TABLE IX

DECADE DISTRIBUTION OF AGE AT ONSET OF SALIVARY CARCINOMATA

Decade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Total*
Male	1	3	2	1	8	9	7	7	1	0	39
Female	1	1	3	0	3	5	8	4	0	0	25
Total	2	4	5	1	11	14	15	11	1	0	64

TABLE X

DECADE DISTRIBUTION OF AGE AT TIME OF FIRST OPERATION FOR SALIVARY CARCINOMATA

Decade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Total*
Male	0	1	1	3	5	10	9	9	1	0	39
Female	0	2	0	2	1	5	9	6	0	0	25
Total	0	3	1	5	6	15	18	15	1	0	64

Like other tumours of salivary tissue, the parotid gland is the commonest site (Sirsat, 1953), followed by the submandibular and sublingual glands, the palate being an intermediate site (Table XI). There would appear, in this series, to be no preference for side, and, as according to rule, bilateral salivary carcinomata were not encountered. In series of malignant salivary tumours there appears to be a slight predominance of males (Table XII) although the difference does not appear to have been as marked in previous reviews.

According to Redon (1953) less than 10% survive five years after receiving treatment. In this series, recurrences were noted in 56 of the 64 cases (Table XIII) and in the remainder, death had occurred in four very shortly after operation due to other causes. The histological study of salivary carcinomata presents little difficulty in diagnosis, as the tumours are invasive from the start. A typical carcinoma of salivary origin is shown in Fig. 12 where it arose in the parotid gland. A statistically significant increase

TABLE XI

SITE DISTRIBUTION OF SALIVARY CARCINOMATA

	Parotid	*Submandibular*	*Sublingual*	*Palate*	*Lips*	*Nose*	*Tongue*
Right	24	3	0	6	1	1	1
Left	23	4	1				
Total	47	7	1	6	1	1	1

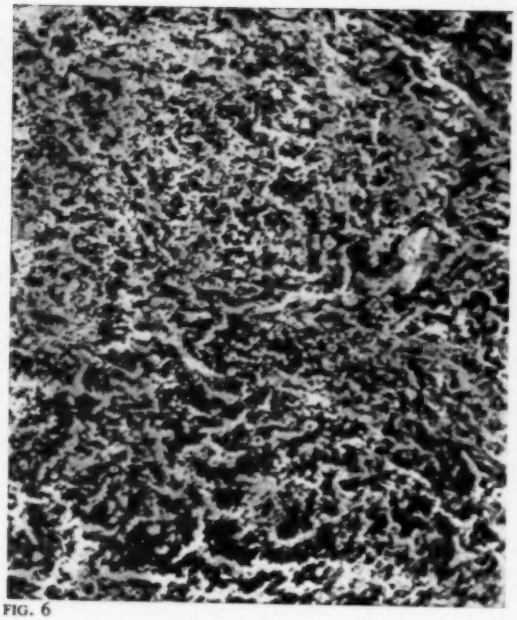


FIG. 6

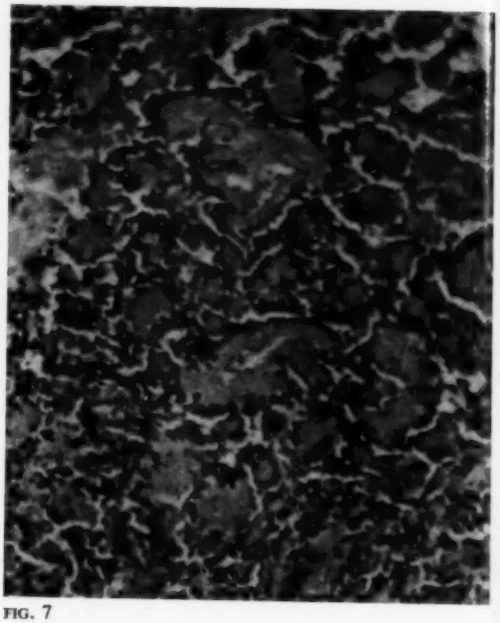


FIG. 7

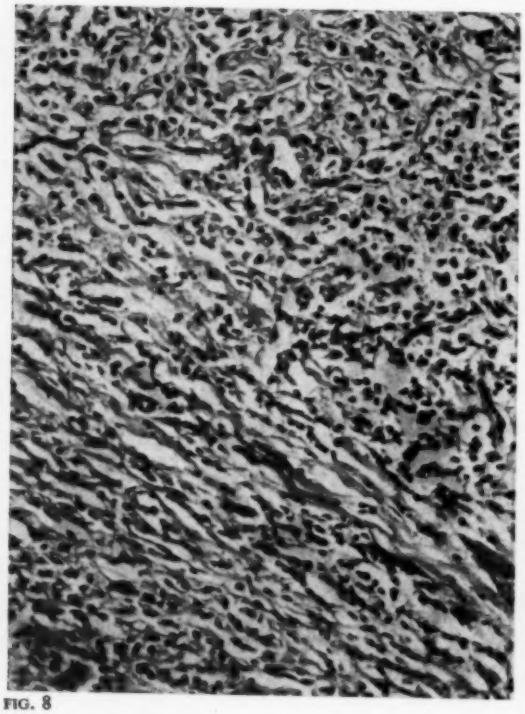


FIG. 8

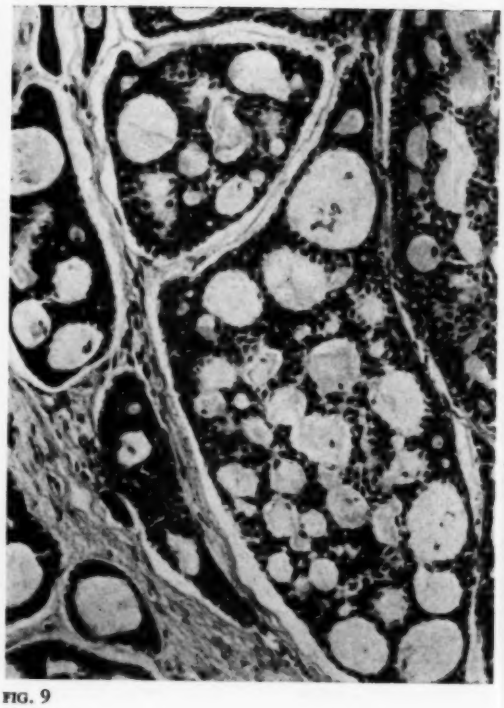


FIG. 9

FIG. 6. *A fairly solid glandular epithelium. Haematoxylin and eosin* × *100.*

FIG. 7. *Photomicrograph of a tubular or cylindromatous type of pleomorphic adenoma. Haematoxylin and eosin* × *180.*

FIG. 8. *A more acinar formation. Haematoxylin and eosin* × *90.*

FIG. 9. *A cribriform pattern. Haematoxylin and eosin* × *190.*

FIG. 10. *Stellate cells in a loose matrix. Haematoxylin and eosin* × *450.*

FIG. 11. *Areas of 'cartilage' in a pleomorphic adenoma. P.A.S.* × *500.*

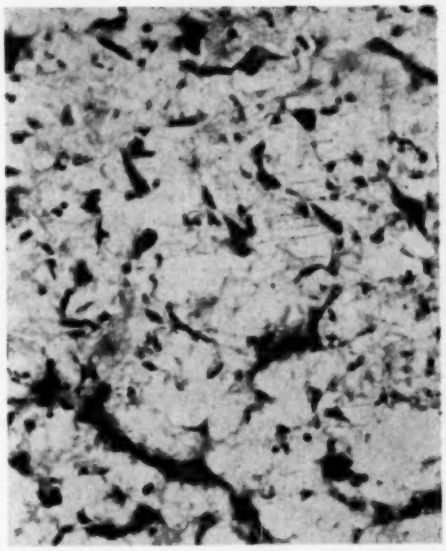


FIG. 10

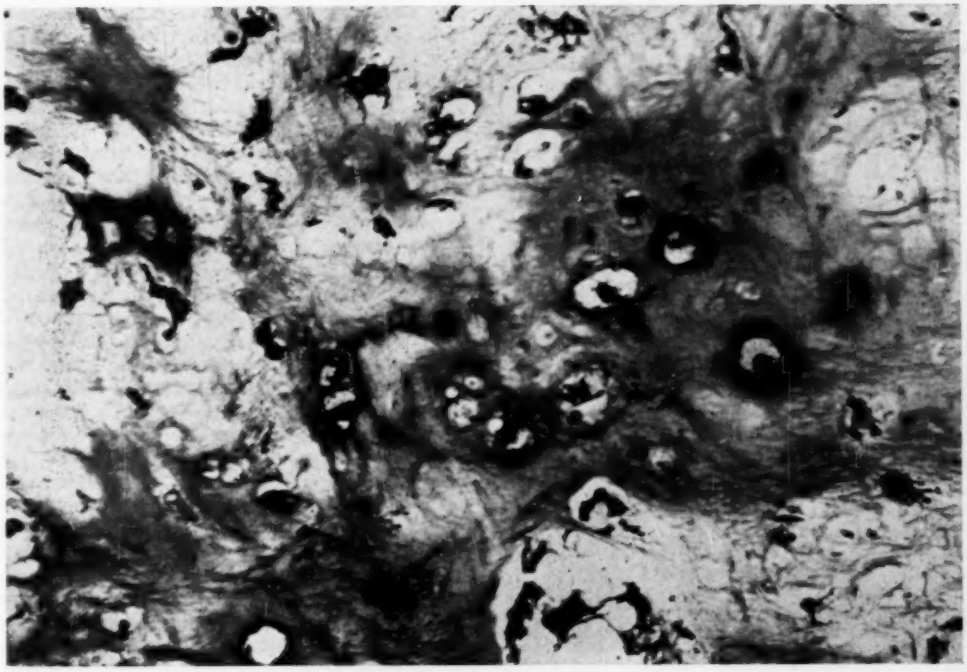


FIG. 11

TABLE XII

SEX DISTRIBUTION OF SALIVARY CARCINOMATA IN THIS SERIES AT SITES OF ORIGIN

	Parotid	*Submandibular*	*Sublingual*	*Palate*	*Lips*	*Nose*	*Tongue*	*Total*
Male	27	6	0	5	0	1	0	39
Female	20	1	1	1	1	0	1	25
Total	47	7	1	6	1	1	1	64

TABLE XIII

RECURRENCE RATE IN SALIVARY CARCINOMATA IN RELATION TO SITE OF ORIGIN

	Parotid	*Submandibular*	*Sublingual*	*Palate*	*Miscellaneous*	*Total*
Recurrences	41	5	1	6	3	56
Non-recurrences	6	2	0	0	0	8
Total	47	7	1	6	3	64

In four of the eight non-recurrences, death occurred shortly after the first operation due to natural causes.

in the number of patients with salivary carcinomata belonging to blood group A was again noted (Cameron, 1958).

TYPE 4: ADENOLYMPHOMA

This group of tumour is of a class unto themselves and occupy about 4% of salivary tumours in this series. Various names have been applied to them, including among others papillary cystadenoma lymphomatosum, branchiogenic cystadenolymphoma, onkocytoma, and Warthin's tumour. They were first reported in 1898 by Hildebrand but were only established as a distinct morphological entity in 1910 by Albrecht and Arzt, being composed mainly of epithelial and lymphoid tissue in or near the parotid. Nicholson (1923) reported the first case in the British literature. As with pleomorphic adenoma various theories as to their origin have arisen, a noteworthy one being that the tumour is branchiogenic, suggested by Ssobolew (1912) and supported by others, although not in vogue today. Another suggested by Warthin (1929), after whom the tumour is sometimes called, is that they arise from the epithelium of embryonical pharyngeal entoderm or the upper respiratory tract displaced to the region of the parotid. Among those supporting this theory are Gaston and Tedeschi (1946), Rawson and Horn (1950), Robinson and Harless (1943), and Kraissl and Stout (1933). A third, more acceptable, theory, originally suggested by Albrecht and Arzt (1910) and recently supported by Harris (1937) and Martin and Ehrlich (1944), is that they represent heterotopic salivary gland rests situated in lymph glands adjacent to or in the parotid. Jaffé (1932) pointed out that such tumours are usually situated on the external surface of the gland although the capsule of the tumours may fuse with the capsule of the gland. He regarded lymph tissue as not an essential part of the tumours but merely as a remnant of a lymph node. Yet another theory was postulated by Thompson and Bryant (1950) who made an extensive study of the subject, and on their findings relate the tumours to 'neoplastic proliferation of the parotid ducts included in lymph glands'. Hamperl (1931) suggested the oncocytes as the site of origin, but Ackerman (1943) considered oncocytomata as a different entity from

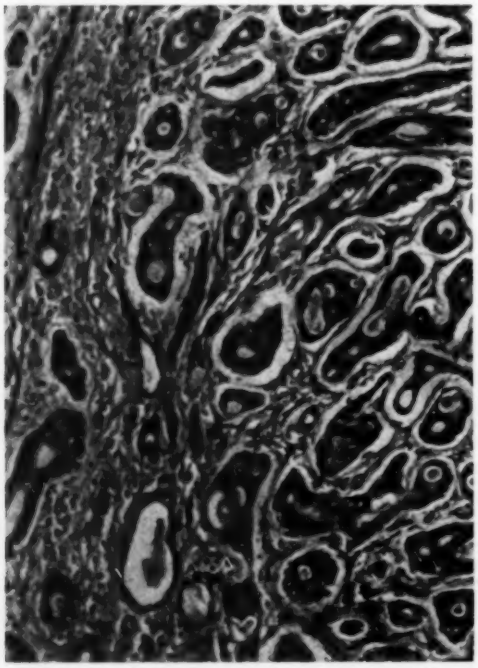


FIG. 12. *Salivary carcinoma of the parotid gland. Haematoxylin and eosin* × *200.*

adenolymphomata. Meza-Chávez (1949), however, showed the occurrence of oncocytes in normal parotid glands.

Adenolymphomata are rarely found in sites other than the parotid gland (Plaut, 1942). In the present series all but one of the 16 tumours occurred in the parotid gland; the one exception occurred in the right tonsil. Only a few bilateral cases have been reported (Nino, 1940; Oughterson, 1941; Ramage, Binnie, and McCall, 1943; Lederman, 1943; Martin and Ehrlich, 1944; Lawrence and Procita, 1948; Foote and Frazell, 1953; and Schulenburg, 1954). In this series, bilateral adenolymphomata were found in four cases; of the remainder, seven occurred in the left parotid and four in the right parotid gland. They can occur at any age, but are generally encountered in the seventh decade. Not a single case was found in the first three decades of life in this series. The age distribution at the onset and at the time of first operation is shown in Tables XIV and XV. Plaut (1942) gives the figure as 84% occurring

TABLE XIV

DECADE DISTRIBUTION OF AGE AT FIRST APPEARANCE OF ADENOLYMPHOMATA

Decade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Total*
Male	0	0	0	1	1	1	3	2	0	0	8
Female	0	0	0	2	1	2	1	2	0	0	8
Total	0	0	0	3	2	3	4	4	0	0	16

TABLE XV

DECADE DISTRIBUTION OF AGE AT TIME OF FIRST OPERATION FOR ADENOLYMPHOMATA

Decade	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Total*
Male	0	0	0	1	1	0	3	2	1	0	8
Female	0	0	0	1	1	1	3	2	0	0	8
Total	0	0	0	2	2	1	6	4	1	0	16

after the age of 40 years. The youngest case recorded is in a boy aged 2½ years (Stöhr and Risak, 1926) and the oldest in a man aged 92 (Carmichael, Davie, and Stewart, 1935). The findings of previous workers that these tumours were commoner in females was not shown in this series (Table XVI). They are well encapsulated and rarely malignant, malignant changes having been recorded in only five cases (Ssobolew, 1912; Stöhr and Risak, 1926; Lederman, 1943; Lloyd, 1946; and Gaston and Tedeschi, 1946).

There were no recurrences noted in any case to date and no case in this series was reported as malignant. The histological pattern of salivary adenolymphomata is characteristic. The tumours are frequently cystic in appearance (Fig. 13) and show a branching papillary structure with intervening collections of small lymphocytes. Fig. 14 shows an

TABLE XVI

SEX RATIO OF ADENOLYMPHOMATA IN PRESENT SERIES COMPARED WITH THAT IN PREVIOUS REVIEWS

Author(s)	*Female*	*Male*	*Ratio*
Carmichael *et al.* (1935)	24	4	6 : 1
Plaut (1942)	50	12	9 : 2
Martin and Ehrlich (1944)	20	2	10 : 1
Gaston and Tedeschi (1946)	73	17	9 : 2
Lloyd (1946)	5	1	5 : 1
Hevenor and Clark (1950)	19	1	19 : 1
Present series	8	8	1 : 1

adenolymphoma with more glandular tissue. In adenolymphomata a statistically significant increase in patients of blood group A was not established (Cameron, 1958) but the indications are that if the number of patients were increased significance would be established.

TYPE 5: MISCELLANEOUS TUMOURS

Numerous tumours have been reputed to have arisen in or adjacent to the salivary glands and in the past have been classified erroneously as sarcomatous tumours of salivary tissue. Other less common tumours to mention only a few, include neurofibroma (McFarland, 1926; Wheelock and Madden, 1949), lipomata (Dockerty and Mayo, 1942), rhabdomyomata (Prudden, 1883; Wood, 1904), leiomyomata (Wheelock and Madden, 1949), angiomata (McFarland, 1926; Wheelock and Madden, 1949), lymphangiomata (Kennon, 1921; Wheelock and Madden, 1949), and malignant melanomata (Rodriguez, 1890; Gilis and Godlewski, 1905; Haggard, 1919).

Haemangiomata, however, deserve brief comment. Usually of the hypertrophic variety and found in infants or children, they are the commonest cause of chronic unilateral parotid swelling in the first year of life. In the present series, only six tumours were included in this category. They included two melanomas, two reticulum cell sarcomas, a recurrent leiomyosarcoma, and a fibroma. All but the fibroma had recurred, and all the patients had died before the follow-up was possible.

CONCLUSIONS

From this survey it would appear that it is only necessary to classify tumours of salivary tissue as pleomorphic adenoma, carcinoma, and adenolymphoma. The type previously classified as simple adenoma is only a variety of pleomorphic adenoma, and so does not need a separate classification. Also the miscellaneous group of tumours hardly enter the survey as they are only rarities and can occur in almost any organ.

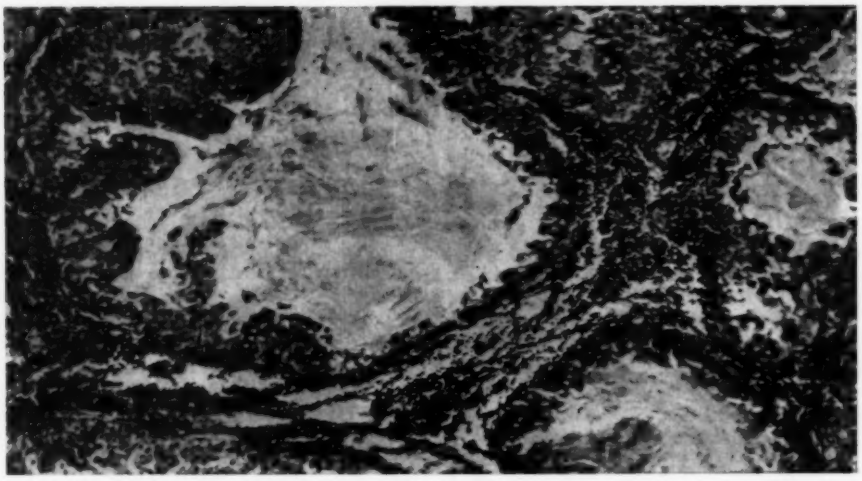


FIG. 13. *Adenolymphoma, rather cystic in appearance. Haematoxylin and eosin × 200.*

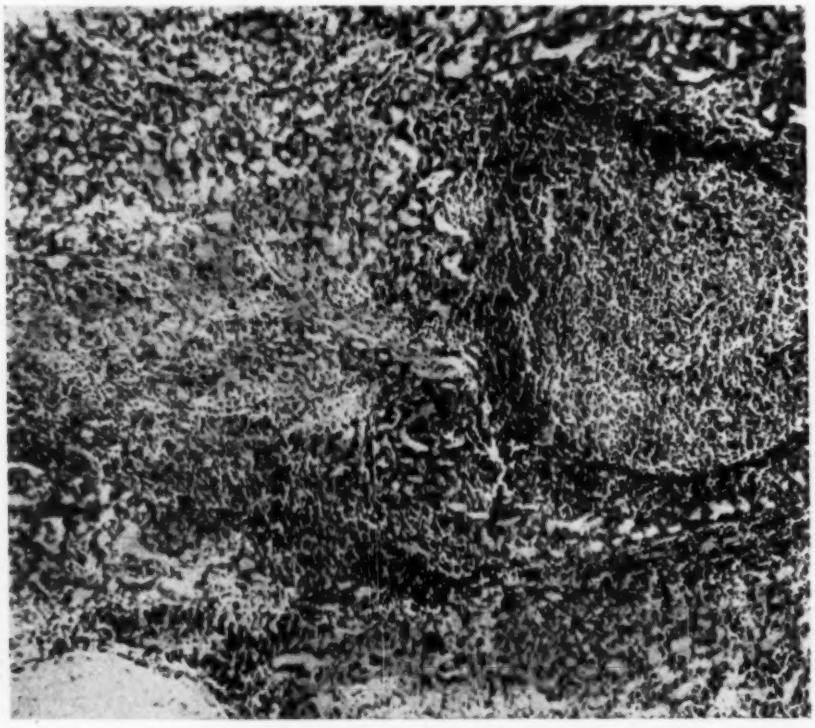


FIG. 14. *Adenolymphoma in more glandular tissue. Haematoxylin and eosin × 100.*

Pleomorphic adenoma form 80% of salivary tumours and 80% of the group occur in the parotid gland with a slight preference for the right side. It was also observed that 60% occurred before 40 years of age and 90% before the age of 60 years. It is agreed that such tumours show a slow intermittent growth and they can reach gigantic size. The recurrence rate of almost 30% is due to either the general habit of such tumours, being subjected to surgery before all the tumour nodules have joined together (?multicentric origin), or that surgery was not radical enough. It is my belief that such tumours should be looked upon as potentially malignant and radically excised.

In the histological study of salivary tumours, numerous sections must be examined in order to get a clear view of the predominant pattern. Much has been written on the presence of areas of 'pseudocartilage', and many deny the possibility of such a finding. In spite of having been unable to prove or disprove this theory by tests *in vitro* with radioactive sulphur (S^{35}) on slices of human salivary tumour, there would appear to be no reason why cartilage, or even bone on rare occasions, should not be seen on histological section. Whether the cartilage is present from the onset of the tumour, or that it arises by metaplastic change will be, for some time to come, a matter for research.

Carcinomata are usually heralded by a tumour showing a rapid increase in size with associated pain and/or paralysis in a patient in a slightly older age group. As would be expected, the recurrence rate is high, and the death rate equally raised.

Only 4% of the tumours in this series were classified as adenolymphoma, and all but one occurred in the parotid gland. According to Carmichael *et al.* (1935) no case of adenolymphoma can be diagnosed correctly before operation; this statement, however, is in my experience, inaccurate.

To Dr. A. Dick for suggesting this subject and together with Professor W. A. Mackey and Professor T. Symington for continued encouragement, criticism, and advice must go my sincere thanks. I am also indebted to Mr. G. F. Headden for his photographic prowess, and Miss H. Scott for secretarial assistance.

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An assessment of the anatomical factor in cor pulmonale in emphysema

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SYNOPSIS The morbid anatomical factors in the production of right heart hypertrophy and failure in emphysema have been reviewed with reference to 44 cases studied by standard histological methods and by post-mortem pulmonary arteriography. The most important factors in raising the pulmonary vascular resistance in these cases are considered to be vascular deformity and pressure by the emphysematous spaces on the adjacent branches of the pulmonary artery. Precapillary bronchopulmonary arterial anastomoses may be of importance in those cases where areas of localized bronchiectasis are present. Intimal fibrosis of the pulmonary arterioles, destruction of the pulmonary vascular bed, and thromboembolic phenomena were thought to be of only minor importance in the production of cor pulmonale in emphysema.

The term cor pulmonale is a somewhat nebulous one and before considering its pathogenesis an accurate definition is essential. The best definition is that given by Mack (1957). He states that 'chronic cor pulmonale may be defined as hypertrophy of the right ventricle resulting from disease involving the lung and/or pulmonary circulation. Failure of the right ventricle need not be present'.

The frequency of this condition is difficult to estimate. White and Brenner (1933) considered that it was rare and accounted for only 1% of patients with heart disease. Wood (1956) concluded that a conservative estimate of its frequency might be 5 to 10% of all cases of organic heart disease. Flint (1954), analysing 300 consecutive cases of congestive heart failure admitted to a general hospital in Sheffield, found cor pulmonale in 76 cases. Whatever the true figure an increase in the incidence of the disease would seem to be likely. Patients suffering from chronic bronchitis and emphysema now receive antibiotics and thus survive to die of heart failure at a later date. Similarly many more children and young adults survive acute pulmonary infections, and the importance of these acute infections in the production of chronic lung diseases in later life has been emphasized by McLean (1957). The use of antibiotics in pulmonary tuberculosis, with subsequent healing by fibrosis, and the increasing number of patients who undergo resection of pulmonary tissue with encroachment on their respiratory reserve, are all factors likely to lead to an increased incidence of right heart hypertrophy and failure secondary to lung disease.

The advent of cardiac catheterization, together with the observation of von Euler and Liljestrand (1946) that anoxia produced a rise in pulmonary artery pressure, has tended to shift attention sharply away from the morbid anatomical lesions in right heart failure due to emphysema. This is unfortunate, as Whitaker (1954) in a careful pathophysiological study of cor pulmonale showed that some factor other than anoxia, high cardiac output, and increased blood viscosity was involved. Whitaker favoured carbon dioxide retention as being of importance but this has not been substantiated. It appears that the importance of the anatomical lesions in this condition has been overlooked. This may be due to the absence of the dramatic histological findings, such as medial hypertrophy and arteritis, seen in isolated histological sections in some cases of pulmonary hypertension secondary to congenital or rheumatic heart disease.

The present paper is concerned with the findings in the pulmonary vessels as shown by conventional histological techniques and by post-mortem pulmonary arteriography. Arteriography gives an overall picture of the pulmonary vascular tree in a manner which is impossible by any other method. The degree of structural damage to the pulmonary arterial system cannot be assessed even on the basis of numerous isolated blocks of lung tissue studied by routine histological sections. If this is true of the

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lung in such conditions as primary pulmonary hypertension and mitral stenosis (Short 1956) it is even more the case in chronic lung disease where the changes are much more likely to be focal in nature.

MATERIAL

CASES STUDIED Forty-four cases coming to necropsy with the diagnosis of chronic bronchitis and emphysema have been examined. Details of the heart size and weight and the morbid anatomical type of emphysema (Ciba Symposium, 1959) from which the patients suffered are given in the accompanying Table. The thickness of the right ventricle was measured at the base and a measurement of 0·7 cm. or greater was considered to denote hypertrophy. The left ventricle was taken to be hypertrophied if it measured 1·5 cm. or more in thickness. All the patients complained of cough, expectoration of sputum, and breathlessness, and most of them died a respiratory death with an acute infection or congestive cardiac failure. Those dying from other causes are listed in the Table.

The thoracic organs were removed from the cadaver in one piece. Considerable care was taken not to puncture the lungs at any point and if necessary the parietal, as well as the visceral, pleura was removed. The heart was dissected from the lungs, the pulmonary veins being cut individually in their intrapericardial course but the pulmonary artery was left intact and was divided from the heart at a point just above the pulmonary valves.

PULMONARY ARTERIOGRAPHY

RADIO-OPAQUE MEDIUM A barium sulphate mixture (Micropaque) was used. The injection mass consisted of 75% Micropaque and 5% gelatin in water. The mixture was heated to, and kept at, a temperature of 75°C.

TABLE

Case No.	*Thickness of Ventricles (cm.)*		*Heart Weight (g.)*	*Type of Emphysema*				*Bronchiectasis*	*Cases Dying from Other than Respiratory Causes*
	Right	*Left*		*Bullous*	*Centrilobular*	*Paracinar Destructive*	*Associated with Focal Fibrosis*		
1	0·9	1·4	540			+			
2	0·7	Not recorded	420		+				
3	1·0	1·6	520		+		+		
4	0·9	2·2	850	+					Systemic hypertension
5	1·0	1·4	520		+		+		
6	0·9	1·2	480		+				
7	0·6	1·8	354			+			
8	0·7	1·6	400		+			+	
9	0·5	1·2	400		+				Myocardial infarct
10	1·2	1·4	300		+				
11	1·0	Not recorded	324		+				
12	1·2	1·2	320			+			
13	0·8	1·5	540		+				
14	1·0	Not recorded	530		+				Myocardial infarct
15	0·8	2·2	536		+		+		Hypertension
16	0·8	1·8	440		+				
17	0·6	1·2	260	+					Head injury
18	0·8	1·8	340			+	+	+	
19	1·0	1·5	530		+	+			
20	0·7	2·1	476		+				Hypertension
21	1·1	2·0	450		+	+	+		
22	0·8	1·2	380			+	+		
23	1·0	1·2	370		+				
24	0·9	1·4	310		+		+		
25	0·8	1·2	540	+					Myocardial infarct
26	0·4	1·6	420	+					Head injury
27	0·8	1·2	402		+		+	+	
28	0·7	1·2	410	+			+	+	
29	0·4	1·3	360		+		+	+	
30	1·0	1·2	382		+		+	+	
31	1·0	1·4	500		+			+	
32	0·9	1·4	390		+	+			Haematemesis
33	1·0	1·4	480			+			
34	0·7	1·2	290		+			+	Carcinoma of stomach
35	1·0	1·8	520		+	+	+	+	
36	0·6	1·6	362		+	+			Head injury
37	0·5	1·2	280				+		Aspirin poisoning
38	1·0	1·6	500		+	+			
39	0·7	1·4	364	+			+		
40	0·8	1·6	416	+			+		
41	1·0	1·6	340		+	+		+	
42	0·9	2·0	540		+	+			
43	1·2	2·0	650		+	+	+	+	
44	1·0	1·5	646		+			+	

before use. This medium penetrates to vessels of 0·03 mm. diameter and gives a satisfactory image on the radiograph.

PREPARATION AND INJECTION OF LUNGS The method described by Short (1956) was used. The lungs were immersed in saline at 40°C. for one hour before injection which was always carried out within three hours of removal from the body. This warming of the lung had the dual effect of relaxing the arteries and ensuring that the injection mass did not set until the fine vessels had been penetrated.

Post-mortem clot was removed by hand from the pulmonary arterial tree in the form of a 'mare's tail'. The pulmonary artery was then flushed gently with warm saline to remove further clot. Full-scale perfusion of the pulmonary artery with saline was not found to be necessary. A cannula with an attached polythene tube was then tied into the main pulmonary artery and the injection was made at a pressure of between 50 and 100 mm. Hg, the aim being to produce complete distension of the pulmonary arteries. The point at which the injection was complete was determined by watching the surface of the lung where the fine vessels in the secondary lobules could be seen through the thin parts of the visceral pleura. The injection mass never appeared in the pulmonary veins but the hilum of the lungs was watched carefully because reflux through the bronchial arteries was seen in cases of bronchiectasis. Histological evidence is needed before an arteriogram can be classed as satisfactory and the arteries said to be fully distended. In the uninjected lung the elastic laminae of the pulmonary arteries appear crenated but in the injected vessels the arterial wall appears much thinner and the elastic coats are smooth. An exception to this occurs when there is severe intimal fibrosis of the arteries which gives the vessels increased rigidity and prevents distension of the elastic laminae.

FIXATION AND RADIOGRAPHY Once the injection had been completed the pulmonary artery was ligated and the cannula removed. The trachea was then connected up to a reservoir containing 10% formol saline and the lung slowly distended with fixative under a pressure of 40 cm. of water. The trachea was then clamped and the lungs immersed in a large bath of formol saline for three days. After this time the lungs were separated and each lung radiographed separately. The lung was then placed lateral (costal) side downwards on a wooden board fitted with Perspex runners, 1 cm. deep, and cut into 1 cm. thick slices using a specially made 17 in. ham knife. These slices were than radiographed.

All radiographs were taken using a mobile Solus *x*-ray machine with attached transformer. Standard Kodak screen-type film was used without intensifying screens, the film being placed in a cardboard container. The anode-film distance was 80 cm. For radiography of whole lungs an exposure of 10 sec. was used at Kv. 85 and Ma. 5. For radiographs of 1 cm. slices an exposure of 4 sec. was used with Kv. 48 and Ma. 5. All films were processed under standard conditions with four minutes in developer at 68°F.

Finally, all the slices were examined carefully together with their corresponding radiographs and suitable blocks of tissue were selected for histological examination. At least 20 blocks were taken from each lung. Sections were stained by haematoxylin and eosin, orcein, van Gieson, and occasionally by the Masson trichrome method.

RESULTS

In most patients dying with chronic bronchitis and emphysema there is a multiplicity of pathological findings in the lungs. Cases of pure centrilobular or pure panacinar destructive emphysema are rare and a mixed picture, with in addition some focal areas of fibrosis and areas of localized bronchiectasis, is nearly always found. It is for this reason that the term 'chronic non-specific lung disease' should be used. The histological appearances of a normal lung and of these various types of emphysema are illustrated in Figs. 1, 2, 3, and 4. Bullous emphysema provides an exception and in the present series there were four cases (4, 17, 18, and 19) where the emphysema took the form of large bullae at the apices and along the anterior borders of the lungs. The rest of the lung parenchyma appeared normal (Fig. 5). In none of these cases was there congestive cardiac failure or right ventricular hypertrophy which could be attributed to the condition in the lungs. The pulmonary arteriograms in these cases were quite normal except for a mild degree of deformity in the region of the bullae. Unless bullae are exceptionally large it is difficult to see how they can exert any effect on the pulmonary artery pressure. In this connexion the report by Stevenson and Reid (1959) of unilateral obliteration of the pulmonary artery is interesting. Unfortunately, no pathological studies have been made on their case but it seems most likely that the right pulmonary artery had been completely compressed by a large bulla or group of bullae. Obviously if such a condition was bilateral and sufficiently extensive, enough of the pulmonary vascular bed could be obliterated to cause congestive cardiac failure.

If the cases of pure bullous emphysema are excluded 40 cases remain and the findings in these are described below.

HISTOLOGICAL CHANGES IN THE VESSELS The *main pulmonary artery* and the pulmonary trunk were studied in each case. Atheroma was present in every case to a greater or lesser degree. It took the form of focal fibrous thickening of the intima with areas where lipoid-containing macrophages were prominent. In some cases the intima was concentrically thickened with fibrous tissue. There was no obvious difference between those cases with right ventricular hypertrophy and those without it. The media of these vessels showed an adult configuration of

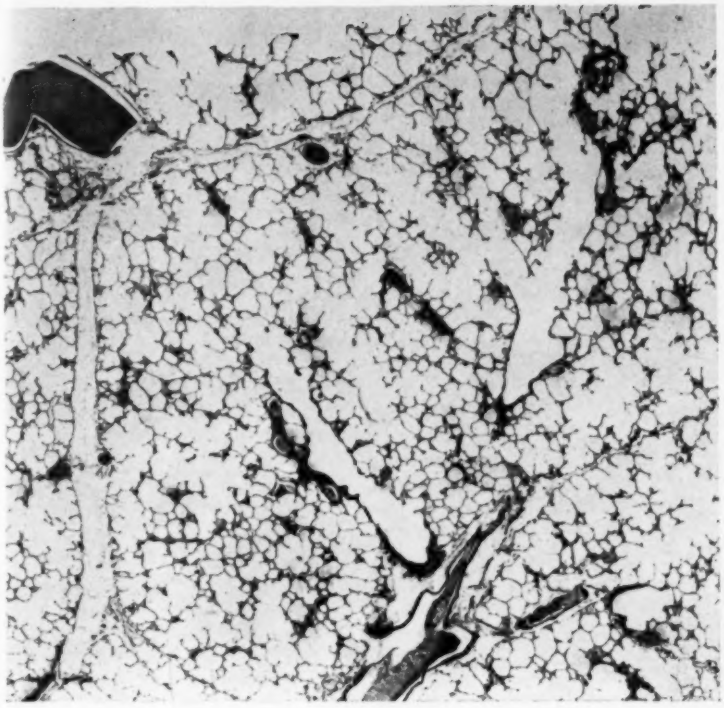


FIG. 1

FIG. 1. *Normal lung showing a secondary lobule. Two terminal bronchioles can be seen which lead into respiratory bronchioles, alveolar ducts, and atria. Pulmonary artery injected. Orcein × 9.*

FIG. 2. *Case 16. Centrilobular emphysema which is fairly extensive. The dilated emphysematous spaces can be seen in the centre of the lobules. Orcein × 6.*

FIG. 3. *Case 33. Panacinar destructive emphysema. The large irregular air spaces extend throughout the entire secondary lobule. Orcein × 4.*

FIG. 4. *Case 28. Irregular emphysema in relation to a focal area of fibrosis which shows a mass of condensed elastic tissue. Orcein × 4.*

FIG. 5. *Case 4. Bullous emphysema. Apart from apical bullae the lung is normal. Pulmonary artery injected.*

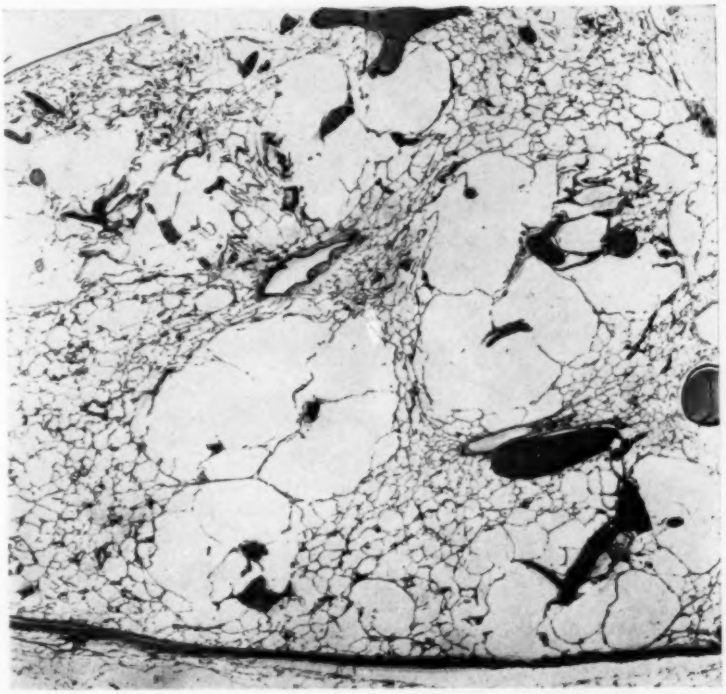


FIG. 2

FIG. 3

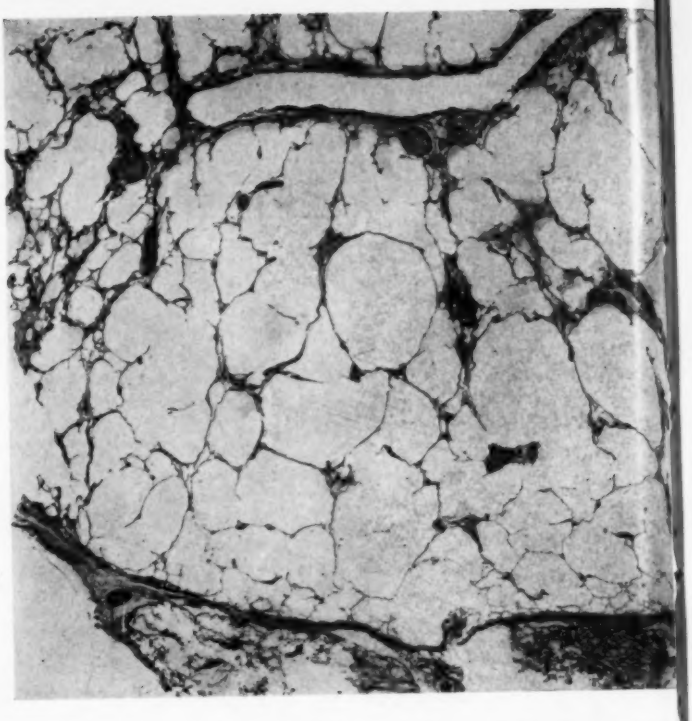


FIG. 5

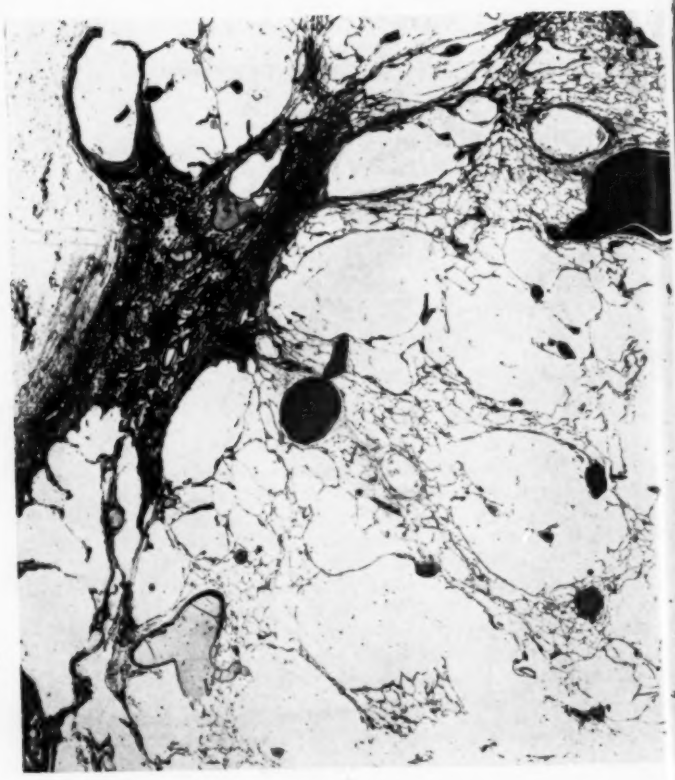


FIG. 4

FIG. 6. *Case 32. Intimal fibrosis in a muscular pulmonary artery adjacent to a dilated bronchiole filled with mucus. Uninjected specimen. Orcein* × *80.*

elastic tissue; in no case was there anything approaching an aortic pattern. The most striking change was the amount of metachromatic material present in azur A preparations. In the cases with right ventricular hypertrophy this was greatly in excess of that seen in cases without right ventricular hypertrophy or in cases of the same age without lung disease.

The *conducting or elastic arteries*, vessels with an external diameter greater than 1 mm., showed similar changes to those seen in the main pulmonary artery in the intima and media. In vessels showing atheroma it was interesting that the elastic laminae were frequently broken up by metachromatic material. In normal vessels of this size the elastic laminae are continuous in contrast to the interrupted laminae in the pulmonary trunk.

The *muscular pulmonary arteries*, vessels with an external diameter ranging from 1 to 0·1 mm., show remarkably little change. Heath and Best (1958) were unable to detect any significant hypertrophy of the media in chronic lung disease even after an exhaustive series of measurements of the total area of the media and comparison with normal controls. Intimal fibrosis was a fairly common finding in muscular pulmonary arteries and was often seen in relation to adjacent diseased bronchioles (Fig. 6). Case 43 was unique in that it did show medial hypertrophy and fibrinoid necrosis in the muscular pulmonary arteries. It has been described fully elsewhere (Dunnill, 1960).

The pulmonary arterioles, vessels of less than 0·1 mm. diameter, showed focal areas of intimal fibrosis in all cases (Fig. 10). This may well pass unnoticed in haematoxylin and eosin preparations but it is clearly apparent in sections stained for elastic tissue. This change is seen as an age phenomenon in normal lungs and so its significance is difficult to assess, but it was noticeable that it was more severe in relation to areas of emphysema.

ARTERIOGRAPHIC FINDINGS The following changes were seen on the arteriographs:—

Whole lung radiographs These are usually completely uninformative as to fine detail but they may

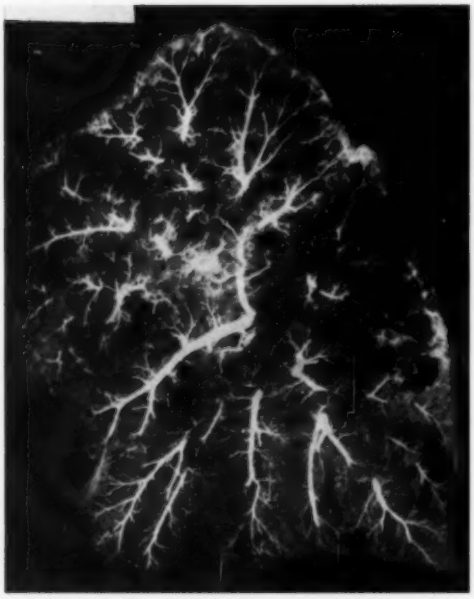


FIG. 7. *Case 38. Post-mortem pulmonary arteriogram. Slice of lung 1 cm. thick showing mixture of centrilobular and panacinar destructive emphysema with destruction of vascular bed. Compare with Fig. 8.*

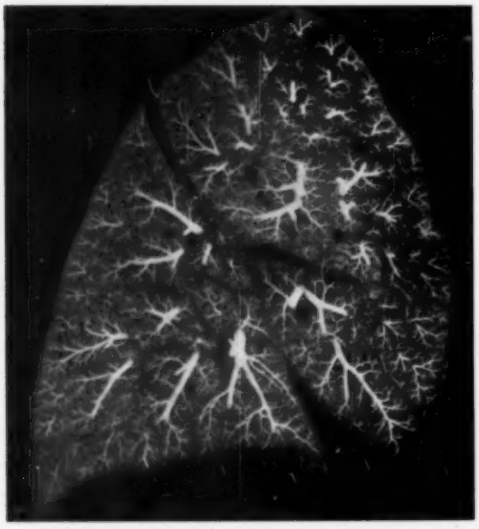


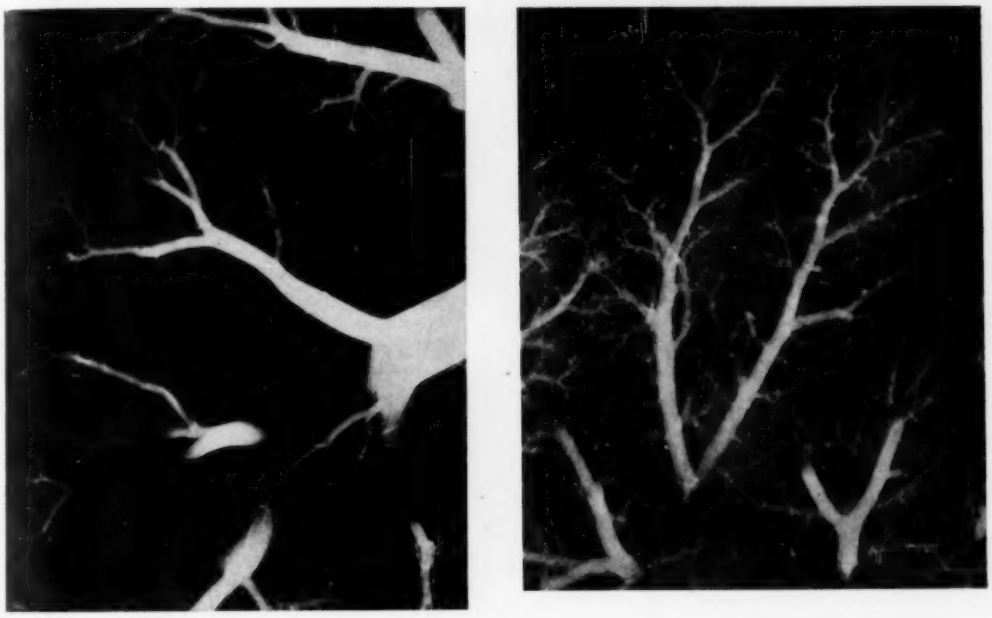
FIG. 8. *Post-mortem pulmonary arteriogram in a man of 40 years with no cardiac or pulmonary disease. 1 cm. slice. The filling defect at the base of the lower lobe is an artefact due to the presence of post-mortem clot.*

show compression and displacement of vessels by apical bullae. The most interesting feature and one that is consistently present is the dilatation of the large hilar, elastic pulmonary arteries when compared with those of a normal lung.

Destruction of pulmonary vascular bed This is the most obvious and most quoted change that occurs in emphysema and it is best appreciated by examination of the radiographs of 1 cm. thick slices of lung (Fig. 7). In centrilobular emphysema, however, destruction of lung tissue may not be very extensive and while it is more so in panacinar destructive emphysema the lung has such a large anatomical and functional reserve that there is seldom enough for this to be an important factor in causing pulmonary hypertension and right heart failure.

Anastomotic channels The presence of precapillary bronchopulmonary arterial anastomoses was demonstrated in all the cases showing localized areas of bronchiectasis. The existence of these anastomoses could often be deduced at the time of the injection by observing that the injection mass was flowing very freely from the cut ends of the bronchial arteries at the hilum of the lung. In many instances numerous small blood vessels on the pleural surface of the lung, covering a bronchiectatic area, were seen to be filled with the injection mass. The subpleural region is an area where the bronchial and pulmonary circulations meet at a capillary level in the normal lung but in bronchiectasis these communications appear to be expanded and enlarged. The main anastomotic networks are, however, in the regions of the bronchiectatic spaces. They were seen most clearly in the radiographs of 1 cm. lung slices but they could also be located on whole lung radiographs. If the diseased area was small the bronchial artery could be clearly visualized taking a spiral course around the long axis of the bronchus in contrast to the pulmonary artery which runs roughly parallel to the bronchus. Where the disease was more extensive the arrangement was disorderly, a whole segment or lobe being the seat of a considerable network of communicating blood vessels.

In no case did the anastomoses occur in the form of a large fistula between a single bronchial and a single pulmonary artery. The anastomoses take place in the smaller vessels situated in the walls of the bronchiectatic sacs. In the normal lung there are capillary communications between the two circulations in the walls of the large bronchi. In localized acute bronchopulmonary infections the bronchial circulation undergoes considerable enlargement in the affected region. It seems likely, as suggested by Liebow, Hales, and Lindskog (1949), that during the formation of granulation tissue in such an area



A B

FIG. 9. *A. Case 22. Close-up of 1 cm. slice showing diffuse narrowing of small branches of the pulmonary artery in an area of panacinar destructive emphysema. B. Close-up of 1 cm. slice of normal lung.*

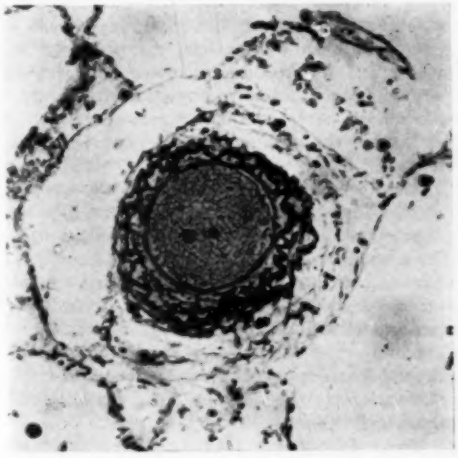


FIG. 10. *Intimal fibrosis in a pulmonary arteriole. The injection mass has filled the lumen but the elastic lamina has maintained its corrugated appearance.*

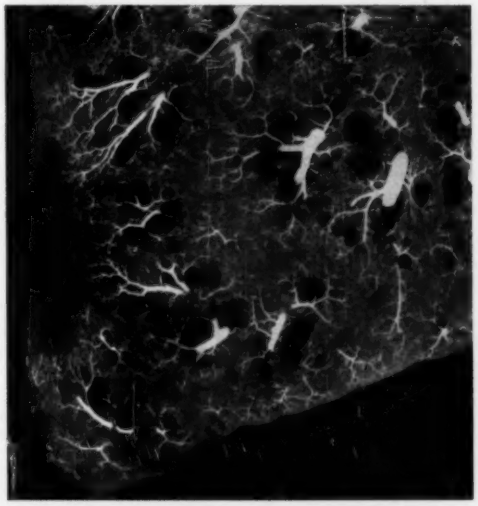


FIG. 11. *Case 44. Deformity of pulmonary arterial branches in an area of centrilobular emphysema in 1 cm. slice. At one border of the specimen there is an area of fibrosis without pulmonary arterial branches.*

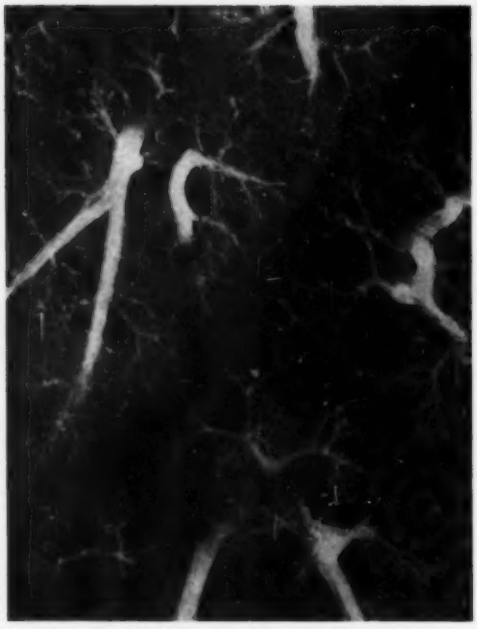


FIG. 12. *Case 33. Close-up of 1 cm. slice in an area of panacinar destructive emphysema. The disease is mainly in the lower lobe; the upper lobe is only slightly affected. The interlobar fissure passes across the centre of the picture.*

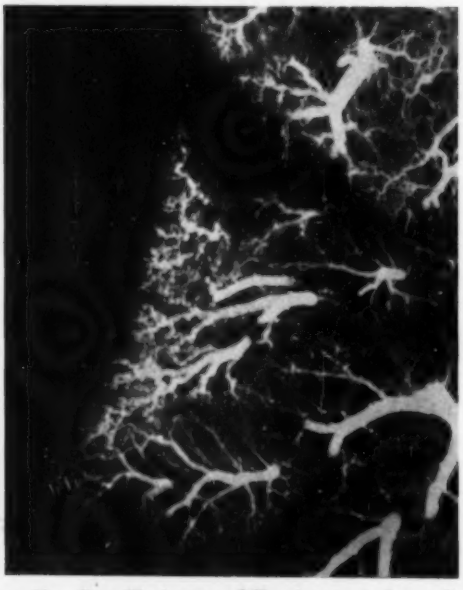


FIG. 13. *Case 37. An area of fibrosis, seen in 1 cm. slice of pulmonary arteriogram, showing the marked tortuosity of branches of the pulmonary artery.*

the two circulations may unite at precapillary level. The actual size of the communications joining the two circulations is greater than 0·03 mm. diameter, beyond which the injection mass will not penetrate, and usually less than 0·1 mm. diameter.

Diffuse narrowing of arteries This change was present in the small arterial branches in areas of emphysema. In radiographs of the whole lung it could be seen as a generalized pruning but it was best demonstrated in 1 cm. thick slices (Figs. 8 and 9). Histological sections of such areas showed that the vessels were fully distended but severely affected by intimal fibrosis (Fig. 10). The lumen was circular and filled with injection mass but the surrounding elastic laminae were corrugated and obviously not stretched to capacity. This is due to the fact that intimal fibrosis results in the arteriole becoming rigid and non-distensible. Frequently there was an 'avascular' area between one secondary lobule and the next. This appearance was due to the fact that the arterioles in these areas were narrowed to less than 0·03 mm. diameter and thus failed to fill with the injection mass.

Areas of centrilobular emphysema These are particularly well shown in pulmonary arteriograms (Fig. 11). They are situated at the point where the artery supplying the secondary lobule bifurcates, the emphysematous space being situated in the fork formed by the two branches. The effect of this on the vessel will depend to some extent on the size of the emphysematous space but even in areas where it is relatively small the adjacent arteries will be deformed. The centrilobular emphysematous space is situated at a key point with reference to the arterial supply to the secondary lobule. If the pressure within the emphysematous space rises the surrounding vessels will be compressed and have to pursue an abnormally circuitous course to the rest of the lobule.

Areas of panacinar destructive emphysema The main arteriographic feature is one of thin spidery arteries which appear much more widely separated than in a normal lung (Fig. 12). The difference is, however, greater than this because in addition very few small branches come off these vessels. This is to be expected from the histological nature of the lesion. The arteriogram represents a combination of parenchymal destruction, with corresponding obliteration of the vascular bed, and intimal fibrosis in the remaining vessels.

Areas of fibrosis Two quite distinct types of vascular pattern were seen in areas of fibrosis. In the first type, which was more frequent, there was a marked degree of tortuosity in the muscular pulmonary arteries and aterioles. This was an arresting finding in the arteriograms (Fig. 13). The

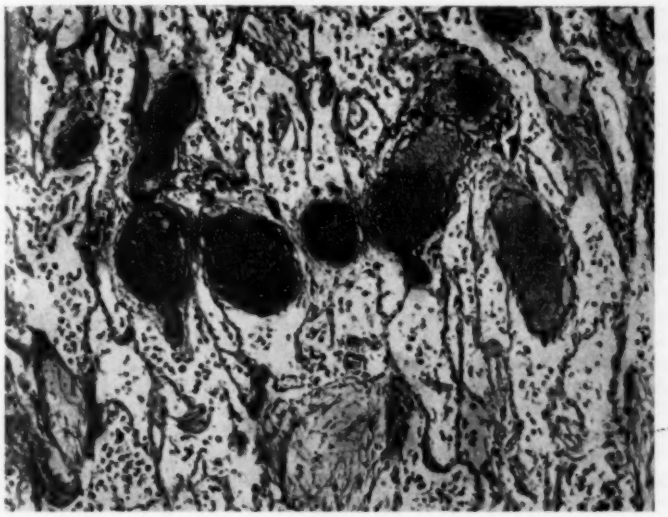


FIG. 14. *Case 37. Photomicrograph of an area of organizing pneumonia and early fibrosis. The pulmonary arterial branches are very tortuous and in a single section one vessel appears many times. Haematoxylin and eosin* × *50.*

histological appearance of these lesions, which were sometimes related to areas of emphysema, depended on their age. A recent lesion appeared as a focus of organizing pneumonia with somewhat shrunken alveoli filled with plump fibroblasts and a few chronic inflammatory cells. In a single histological section of such an area any given artery may appear several times (Fig. 14) thus giving the illusory appearance of increased vascularity. In an older lesion the vascular pattern remains similar but the histology alters because fibrosis is more advanced and the most prominent feature is the dense aggregation of elastic fibres which outline the collapsed alveolar pattern. In this type of lesion the vessels still appeared tortuous but there was very considerable luminal narrowing due to fibrous intimal proliferation.

The second pattern in areas of fibrosis was one of simple avascularity as far as the pulmonary arteriograms were concerned (Fig. 11). The surrounding vessels were thin and tapering. Histology of such an area showed hyaline fibrous tissue surrounded by lymphocytes. Occasionally a giant cell of the Langhans type could be seen and it is probable that these areas, which are not common, represent old tuberculous lesions. This would be in keeping with the views of Cudkowicz (1952) who found that tuberculous lesions frequently gained their blood supply from the bronchial as opposed to the pulmonary circulation.

Antemortem thrombus in branches of pulmonary artery This was a finding in 16 cases. In 12 cases thrombus was found in the femoral veins or deep calf veins at necropsy but in the remaining four cases no systemic venous thrombosis was detected at all. Gross examination of the lung slices in conjunction with the arteriograms showed thrombus in the elastic pulmonary arteries in 10 cases. In the further six cases thrombus was found in the muscular pulmonary arteries when the lungs were examined histologically. It must be assumed that in those cases where leg vein thrombosis was present the thrombus found in the pulmonary arterial branches was embolic in origin. In the four cases where no leg vein thrombosis was found it seems most probable that there was a thrombosis in the small pulmonary arterial branches which took place as a reaction to the adjacent inflammatory reaction in the bronchioles and pulmonary tissue.

In three cases there was evidence of pulmonary infarction. The reason for this is not fully apparent as in none of these cases was the thrombus particularly large nor did the affected lung suffer from chronic passive venous congestion. However, Torack (1958) described pulmonary infarction occurring in the absence of congestion due to heart disease where there was pleural effusion, pneumonia, pulmonary collapse, or bronchial occlusion. In all three cases showing infarction there was coexistent acute pulmonary infection and this was probably the important factor.

DISCUSSION

The syndrome of 'chronic bronchitis and emphysema' is the commonest and most important cause

of cor pulmonale in Great Britain. The factors involved in the pathogenesis of this condition can be divided into disturbances of physiology, such as anoxia, carbon dioxide retention, and polycythaemia, and disturbances in the anatomical structure of the lung and pulmonary circulation. While it is realized that the first group of factors is very important it is with the second group that this discussion is concerned.

INTIMAL FIBROSIS OF ARTERIES AND DESTRUCTION OF PULMONARY VASCULAR BED The normal pulmonary vascular bed is capable of an 'extreme degree of distensibility. Thus in a normal subject the pulmonary blood flow of approximately 5 litres a minute at rest can be increased to values as high as 25 or 30 litres a minute during exercise with little or no increase in pulmonary artery pressure. Factors decreasing this distensibility are of importance in the production of cor pulmonale. It is usually claimed that intimal fibrosis in the arterioles, destruction of the capillary bed, and parenchymal fibrosis are the three most important lesions in this respect. The significance of intimal fibrosis of the arterioles is in dispute. That such lesions occur and are frequent is not doubted but what part they play in raising the pulmonary artery pressure is debatable. Evans (1951) considers them all important. However, intimal fibrosis has been described as a normal age phenomenon in adults with no evidence of cardiac or pulmonary disease by Welch and Kinney (1948) and Civin and Edwards (1951), and, although it may be more extensive in cases of emphysema (McKeown, 1952), the difference is not enough to account for right ventricular hypertrophy. The origin of the intimal fibrosis is unknown. It may represent organized mural thrombus, and McLean (1958) has pointed out that there is a close topographical relationship between sclerotic arterial lesions and lung tissue showing chronic inflammatory damage. In particular he observed that sclerosis was much more striking in arteries adjacent to diseased bronchioles and he concluded that in many cases this intimal fibrosis represented organization of mural thrombus which had formed as a reaction to past acute inflammation. In general it may be concluded that these sclerotic arterial changes in emphysema are never sufficiently widespread or advanced to cause pulmonary hypertension on their own account. They differ too little from the normal age change and their distribution is too localized.

Mack (1957) considers that destruction of the pulmonary vascular bed by conversion of small air spaces into fewer larger spaces with destruction of alveolar septa and loss of capillaries is important in the production of cor pulmonale in emphysema. However, before there is any rise in pulmonary artery pressure there must be a considerable degree of destruction of pulmonary tissue. Flores, Adams, and Perkins (1954) reduced the pulmonary arterial tree of dogs to 15% of normal without producing pulmonary hypertension at rest though they did find a rise in pressure on exercise. In clinical practice numerous patients have undergone resection of a lung without developing pulmonary hypertension, at least during a short-term follow-up. Motley (1959) has found that pulmonary heart disease may develop in the older age groups after a latent period of several years during which regular work and activity have been carried out. He considers that heart failure in these cases is due to the over-distension of the remaining lung which takes place to fill the space left by the tissue which has been removed, and that the condition can be prevented by thoracoplasty.

In the various forms of emphysema destruction of the pulmonary vascular bed probably plays a minor rôle in the pathogenesis of cor pulmonale. In many cases of centrilobular emphysema with right ventricular hypertrophy the pulmonary destruction is confined to the centre of the secondary lobules, the alveoli at the periphery being relatively normal. In panacinar destructive emphysema the degree of destruction of the vascular bed is more striking but it is seldom enough to force one to conclude that it is a major factor in raising pulmonary vascular resistance when it is remembered that, if an analogy can be drawn from experimental work, over 80% of lung tissue would have to be destroyed.

AREAS OF FIBROSIS Focal areas of fibrosis are common in chronic non-specific lung disease. Numerous authors (Mack, 1957; Motley, 1959; Spain, 1959) imply that in areas of pulmonary fibrosis there is vascular obliteration with narrowing of the vessels due to contraction of surrounding fibrous tissue. This may be seen in a few areas but more commonly the areas of fibrosis contain branches of the pulmonary artery that are most remarkable for their tortuosity. This phenomenon of vascular deformity with increased tortuosity as a factor in raising pulmonary vascular resistance was emphasized by Patel and Burton (1957). They found deformity or 'gnarliness' of the pulmonary arterial branches in rabbits following the injection of norepinephrine and they considered it to be a major factor in raising the pulmonary vascular resistance and thus the pulmonary artery pressure. Similarly Reynolds (1956) has shown that in guinea-pigs the raised pulmonary vascular resistance during foetal life could be correlated with the coiled and tortuous nature of the small pulmonary vessels in the foetal lung. After birth when the pulmonary vascular resistance falls dramatically these vessels uncoil and become straight.

Clearly in emphysema the areas of fibrosis with their tortuous vessels are of no great importance in raising the pulmonary vascular resistance as a whole unless they are very extensive. Their main importance lies in the fact that they raise the resistance locally to pulmonary blood flow thereby serving to divert blood away from the fibrotic areas to the more normal parts of the lung where oxygenation can take place.

AREAS OF LOCALIZED BRONCHIECTASIS The significance of precapillary bronchopulmonary arterial anastomoses in the production of right ventricular hypertrophy in bronchiectasis has been fully discussed by Liebow *et al.* (1949). These anastomoses raise the pulmonary artery pressure and increase the work of the right ventricle, because the pressure in the bronchial arteries is related to the pressure in the aorta and is therefore higher than that in the pulmonary artery. In the cases discussed here the areas of bronchiectasis were small and localized and probably played only a minor rôle in the production of right ventricular hypertrophy. It is interesting to note that Spain (1959) considers that cor pulmonale does not occur in bronchiectasis unless the disease is diffuse, bilateral, and accompanied by emphysema.

It should be noted here that no attempt has been made in this series of cases to demonstrate the bronchopulmonary venous anastomoses described by Liebow (1953) in cases of emphysema. It appears that most of his patients suffered from bullous emphysema rather than one of the more diffuse varieties of the disease.

THROMBO-EMBOLISM Thrombo-embolic phenomena are common in the pulmonary arteries of patients with pulmonary emphysema. There is little evidence, however, that they play a major part in the production of pulmonary hypertension in this condition. Kernen, O'Neal, and Edwards (1958) reviewed the incidence of pulmonary arteriosclerosis and thrombo-embolism in emphysema. They concluded that neither could be an important factor in the pathogenesis of pulmonary hypertension with chronic emphysema since the incidence of both pulmonary arteriosclerosis and thrombo-embolism was the same in cases of emphysema with right ventricular hypertrophy and without right ventricular hypertrophy.

AREAS OF CENTRILOBULAR EMPHYSEMA Leopold and Gough (1957) in their original communication on the centrilobular form of emphysema noted that right ventricular hypertrophy was present in over half their cases. In the present series of cases the relationship was even more marked. The explanation for the right ventricular hypertrophy in these cases is not at once apparent. In many instances the destruction of the vascular bed is not great, the areas of emphysema being confined to the centre of the lobule, fibrosis is not a prominent feature, and the degree of intimal thickening in the small branches of the pulmonary artery is not enough to account for the cardiac hypertrophy. A possible answer is to be found in the pulmonary arteriograms. Attention has been drawn to the fact that centrilobular emphysematous spaces are often situated at the bifurcation of the muscular pulmonary arteries supplying the secondary lobules. The arteries instead of pursuing a straight course curve around the emphysematous space bringing in an element of vascular deformity. Probably the most important factor in these cases is the pressure of the emphysematous space itself on the adjacent artery. In considering resistance to flow in arteries pressure on the vessels from without is usually ignored, attention being focused almost entirely on thrombus in the lumen and intimal fibrosis and medial hypertrophy in the wall. In emphysema, where medial hypertrophy is seldom if ever present and thrombus and intimal fibrosis are insufficient to account for the cardiac changes, it may well be that this extravascular factor is all important. Spain and Handler (1946) considered that intra-alveolar pressure on the pulmonary capillaries was important but they appear to have given little attention to the arteries.

Physiological studies bear out the importance of intra-alveolar pressures on pulmonary blood flow. Bjurstedt and Hesser (1953) showed that in dogs during inspiration the output of the right heart increases and there is an increase in pulmonary artery pressure. García Ramos (1955) found that in normal animals the pulmonary capillaries were flattened during inspiration and this has been thought to be the explanation for the increase in pulmonary vascular resistance during this phase of the cycle. Riley (1959), using isolated lung preparations, showed that there was an increase in resistance to pulmonary blood flow during lung inflation. In emphysema the findings are somewhat different. Attinger, Goldstein, and Segal (1956) and Dekker (1958) have demonstrated clearly that in emphysema expiration is very prolonged with the production of a markedly positive intra-alveolar pressure. The measurement of absolute values is subject to error due to technical difficulties but it is possible for values of at least 40 cm. of water to be sustained during expiration. Such pressures when exerted on the smaller branches of the pulmonary artery will easily cause compression and occlusion; the pressure in the main pulmonary artery does not reach these levels during health. Although such a pressure is only intermittently exerted it must be remembered that in emphysema, where the expiratory part of

the cycle may be as much as five times as long as the inspiratory component, the vessels will be subject to this extramural pressure for a very considerable time. Furthermore during the expiratory phase blood will still be pumped into the lungs and, if it cannot penetrate to the alveolar capillaries, the elastic, conducting pulmonary arteries will dilate. This dilatation of the larger pulmonary arteries has been noted in this series and also by Harrison (1958) and Meessen (1959).

One of the interesting facts about the pulmonary artery pressure in emphysema is that it is often normal or only slightly raised when the patient is not in cardiac failure. In failure this pressure rises considerably but is not due entirely to anoxia (Whitaker, 1954) although attacks of congestive failure are frequently precipitated by acute pulmonary infections. During such an infection it is the bronchioles which are most severely affected with peribronchiolar inflammation and exudation of mucus and pus into the lumen of the air passages. It is this type of exudate that may well constitute a ball-valve obstruction, air entering the peripheral lung with powerful inspiration but expulsion of the air being difficult with weaker expiration. The intra-alveolar pressure will rise and will be felt particularly by the weakened centrilobular spaces rather than the relatively normal and more distal alveoli. Furthermore this raised pressure will be transmitted to the surrounding branches of the pulmonary artery, serving to raise the resistance in these vessels.

The situation of the centrilobular emphysematous spaces near the apex and in the centre of the secondary lobule is important. It is at the apex that the arterial supply enters the lobule, thus pressure on the vessels at this point will be most effective in raising resistance to blood flow in the whole area of the lobule. Probably it is because of this that in many cases of widespread centrilobular emphysema, in which many lobules are affected to a minor degree, right ventricular hypertrophy is present. There may be little parenchymal destruction but that which is present occurs at a vital point.

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Nocardiosis in anaemic patients given steroids

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SYNOPSIS Two fatal cases of infection with *Nocardia asteroides* are described with necropsy findings in one. Both patients had been anaemic and had received steroid therapy.

The bacteriological problems of species recognition in the genus *Nocardia* are considered and the results of infection in experimental animals described.

Nocardia asteroides rarely produces human infection in Britain although the infection is not uncommon in the United States of America (McQuown, 1955). In the majority of such cases pulmonary lesions occur and the respiratory tract is assumed to be the portal of entry (Ballenger and Goldring, 1957). Infection may be disseminated to the brain causing cerebral abscesses; *Nocardia asteroides* was first isolated from a cerebral abscess by Eppinger in 1890.

Two cases are reported; in both the mode of infection is debatable. The patients suffered from severe anaemia and were treated with steroids; these may have been predisposing factors.

CASE REPORTS

CASE 1 A man aged 73, a retired hedger, was admitted in July 1958 with nine months' history of jaundice and fatigue. He was very pale, slightly jaundiced, and the spleen was moderately enlarged.

On admission Hb was 6·0 g. per 100 ml. of blood. Reticulocytes were 29% and W.B.C. 8,500/c.mm. (polymorphs 6,800, lymphocytes 1,300, monocytes 400). The plasma bilirubin level was 1·9 mg./100 ml. The blood group was O Rh positive. The sternal marrow showed marked hyperplasia of the erythroid series. The direct sensitization test (Coombs) was positive and a 'cold' antibody was found in the serum.

A diagnosis of acquired haemolytic anaemia of the 'autoimmune' type was made and prednisone, 15 mg. t.d.s., started. The haemoglobin rose steadily to 10·6 g. per 100 ml. in approximately four weeks and the patient was discharged on prednisone, 25 mg. daily.

Three months after the first admission he presented with painful swellings of the left loin and shoulder which appeared to be acute pyogenic abscesses. The temperature was 102°F. and the W.B.C. 24,000/c.mm. with a polymorphonuclear leucocytosis. Both abscesses were incised; that in the left loin extended between the muscle layers of the abdominal wall into the left peripheric fat; the abscess in the left shoulder was limited to the subcutaneous tissues. Several ounces of thick yellow pus were drained from both abscesses. Smears of pus showed a Gram-positive filamentous organism subsequently identified as *Nocardia*. Chloramphenicol, 1 g. daily, was given for a week. Further abscesses appeared in the subcutaneous tissues of the right calf and left thigh; these were incised and cultures of the pus grew *Nocardia*. Treatment with sulphadiazine, 4 g./day, was begun and continued until the patient's death.

The haemolytic process appeared to escape control with the appearance of the first abscess, the blood haemoglobin fell to 6 g. per 100 ml., and there was only transient improvement with blood transfusion and an increased steroid dosage. Five months after the second admission another abscess in the left loin was drained. *Proteus vulgaris*, *Bact. coli*, and non-haemolytic streptococci were grown from the pus; *Nocardia* was not isolated. Shortly after this the patient had a series of generalized convulsions. Examination of the central nervous system showed no abnormality; the cerebrospinal fluid was normal.

Deterioration continued, the haemoglobin fell to 4·5 g. per 100 ml., and the patient died eight months after he was first admitted.

Necropsy (*P59/191*) An abscess in the left psoas, 2 cm. in diameter contained yellow-grey material with much surrounding fibrosis.

A thin-walled abscess (3 cm. diameter) was present immediately to the right of the superior sagittal sinus, 3 cm. from the confluence of sinuses. The superior sagittal sinus was obliterated in the region of the abscess by organizing thrombus. Section of the brain showed two abscesses in the right fronto-parietal region.

The heart was of normal size; the cardiac muscle showed tabby-cat striation. Grey-yellow vegetations (up to 0·5 cm. diameter) were present on the atrial aspects of the mitral and tricuspid valves and the edges of the aortic valve cusps were thickened by similar vegetations.

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The spleen was moderately enlarged (320 g.), with a soft purple cut surface. A piece of spleen turned blue in acid ferrocyanide solution. The marrow was red and gelatinous in appearance and extended as far as the middle of the right femur; similar marrow was present throughout the vertebral column, and was increased in the diploe of the skull. Lymph nodes were not enlarged.

The kidneys showed no significant abnormality other than sulphonamide crystals in the renal pelves; these were also seen in the bladder.

The right pleural cavity contained 100 ml. of cloudy yellow fluid; the lungs were oedematous but showed no evidence of infection or of a source for the nocardial infection.

The liver was slightly enlarged (1,630 g.) and pale yellow. The oesophagus, stomach, and small and large intestine were normal.

No abnormality was found in the endocrine glands.

Histological findings The parasagittal abscess showed Gram-positive filaments and had a wall of thick fibrous tissue. The cerebral abscesses were filled with a chronic purulent exudate, the wall consisting of glial fibres and abundant astrocytes. Small abscesses containing Gram-positive coccoid organisms were seen in the renal cortex.

Mitral and tricuspid vegetations consisted of clumps of fibrin mingled with clusters of oval coccoid micro-organisms.

The red pulp of the spleen was moderately congested; sinusoids contained an excess of polymorphonuclear leucocytes and many haemosiderin deposits were seen. Haemosiderin was also present in moderately large amounts in the sections of liver. The marrow was of rather low cellularity with an excess of primitive cells but few late normoblasts; deposits of haemosiderin were abundant.

CASE 2 A man aged 67, a retired insurance manager, was first seen in November 1957 with 18 months' history of spontaneous bruising. Examination disclosed moderate enlargement of the liver and spleen but no lymphadenopathy.

On admission Hb was 10·8 g. per 100 ml. of blood, W.B.C. 9,800/c.mm. (polymorphs 7,300, lymphocytes 1,400, monocytes 540). Blood films showed a few late normoblasts and myelocytes in the peripheral blood. Platelets numbered 30,000/c.mm. Hess's test was positive. The bleeding time (Ivy) was 10 minutes, and the clotting time four minutes. A left iliac crest biopsy showed normal marrow cells diluted with blood; further biopsy was postponed because of bleeding from the puncture site.

A provisional diagnosis of myelofibrosis was made and prednisone, 20 mg. t.d.s., started. Ten days later the platelets had risen to 120,000/c.mm. and the W.B.C. to 26,000/c.mm. (polymorphs 21,000).

Prednisone (5 mg. t.d.s.) was continued with the patient coming as an outpatient, and he continued well with only slight bruising until October 1959. During this time the W.B.C. rose gradually to 37,000 per c.mm. and platelets varied from 35,000 to 180,000/c.mm.

He was readmitted in September 1959 with six weeks' history of a painful swelling of the left buttock which appeared to be an acute pyogenic abscess.

On this occasion Hb was 9·7 g. per 100 ml. and W.B.C. numbered 60,000/c.mm. (polymorphs 43,000), myelocytes 10,000, lymphocytes 4,700, monocytes 1,000). Platelets were 80,000/c.mm. The abscess was incised and 8 oz. of thick yellow pus obtained from it. Sulphadimidine, 1 g. four hourly, was started.

Cultures of the pus grew *Nocardia*. Postoperatively the patient's condition deteriorated; the Hb fell to 6·1 g. per 100 ml., urine output fell, and the blood urea rose to 110 mg./100 ml. He became semiconscious and died on 10 October 1959. Permission for a necropsy was refused.

BACTERIOLOGY

MORPHOLOGY The pus obtained from the abscesses of both patients was thick, yellow, and odourless. Direct examination of films of the pus showed a filamentous Gram-positive organism which branched and stained irregularly. The organism was weakly acid fast and on subculture tended to break up into coccoid forms and show a beaded appearance.

CULTURAL CHARACTERISTICS Growth occurred on most ordinary media, including nutrient and blood agar, Sabouraud's medium, Lowenstein-Jensen slopes, nutrient and glucose broth. Of these blood agar was most useful. All original cultures yielded a pure growth. On blood agar incubated aerobically minute grey colonies embedded in the medium were

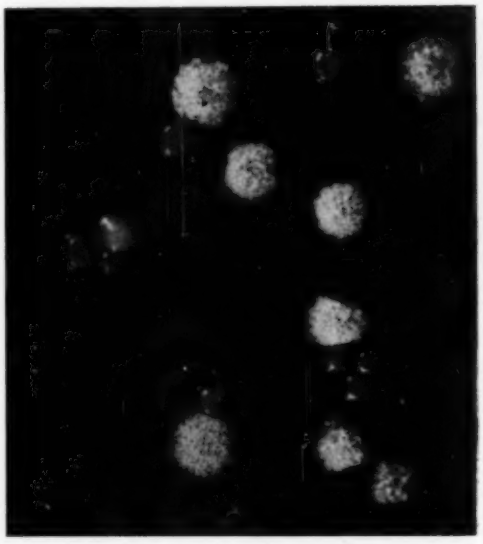

FIG. 1. *Colonial variation of* Nocardia asteroides.

just visible in 24 hours. With continued incubation, for 48 to 96 hours, two types of colony could be distinguished: smooth, domed colonies 1-2 mm. diameter, and colonies of similar size with a rough powdery surface, pale yellow or white (Fig. 1). With continued incubation all colonies became taller and developed a rough surface. After incubation for a week the rough surface could be seen to be due to the development of aerial hyphae. Old cultures become deeply pigmented, usually red but occasionally yellow. Growth did not occur anaerobically. Cultures left on the bench grew quite well but growth was most rapid at 30-40°C.

BIOCHEMICAL REACTIONS Urea was split on Christenson's media by both organisms. Slight and inconsistent fermentation occurred in peptone water sugars. In an attempt to obtain specific biochemical reactions the methods of Gordon and her colleagues (Gordon and Smith, 1955; Gordon and Mihm, 1957, 1959) using sugars incorporated in agar, containing inorganic salts only, were used. The results (Table I) were similar to those of Gordon and Mihm who tested 98 strains.

TABLE I

BIOCHEMICAL REACTIONS OF SPECIES OF NOCARDIA ISOLATED FROM CASES 1 AND 2

	Case 1 After Incubation		*Case 2 After Incubation*	
	8 Days	*28 Days*	*8 Days*	*28 Days*
Decomposition of:				
Casein	−	−	−	−
Gelatin	+		−	−
Tyrosine	−	−	−	−
Acid from:				
Arabinose	−	+	−	+
Galactose	−	+	−	+
Glucose	+	+	+	+
Inositol	−	−	−	−
Lactose	−	−	−	Weak +
Maltose	−	Weak +	−	+
Mannitol	−	−	−	−
Raffinose	−	Weak +	−	−
Rhamnose	+	+	−	−
Sorbitol	−	−	−	−
Xylose	−	−	−	−

ANTIBIOTIC SENSITIVITIES Strips of filter paper soaked in solutions of antibiotics were incorporated in nutrient agar plates for testing sensitivities. Organisms of known sensitivities were used as controls. Both organisms were resistant to penicillin and erythromycin and sensitive to chloramphenicol and sulphamezathine. The organism from Case 1 was resistant to streptomycin and tetracycline, that from Case 2 was sensitive to these two antibiotics.

SEROLOGICAL INVESTIGATIONS No agglutinating or complement-fixing antibodies could be found in sera from Case 1 using live and formalized suspensions of the organism.

COMMENT ON BACTERIOLOGY

The classification of the *Actinomycetaceae* is still unsettled. Waksman and Henrici (1943) divide the aerobic actinomycetaceae into *Nocardia*, in which the mycelium breaks up on subculture, and streptomycetae, in which the mycelium remains intact. Both organisms described in this paper fulfil this definition for *Nocardia*.

N. asteroides is the most common member of the genus; it is acid fast, whereas other *Nocardia* and *Streptomycetae* are not acid fast or only occasionally acid fast (Mariat, 1958), and give the biochemical reactions described by Gordon and Mihm. Both organisms are therefore provisionally identified as *Nocardia asteroides*.

ANIMAL PATHOGENICITY Rabbits and guinea-pigs were infected with the organisms from Cases 1 and 2 in order to study the type and distribution of the lesions produced and also to determine the effect of intramuscular prednisone on the course of such infections.

The organism was suspended in physiological saline producing an even suspension of opacity equivalent to Brown's tube 10. Rabbits were injected with 0·1 ml. intravenously. Groups of guinea-pigs received 0·1 ml. intracardially, under ether anaesthesia, or 0·1 ml. intraperitoneally, or 0·1 ml. intradermally. One batch of the last group was also given prednisone intraperitoneally in comparable doses to those used in man.

Rabbits died in about four days with elevation of the blood urea (200 mg./100 ml.) and those guinea-pigs infected by means of the intracardiac route died in about two days. Histological examination revealed multiple small abscesses composed largely of polymorphonuclear neutrophil leucocytes with a few histiocytes in the lungs, choroid plexus (Fig. 2), myocardium, liver, spleen, skeletal muscles, and in other tissues. Organisms were found with difficulty in the lesions, although cultured with ease. Often they appeared as chains of cocci, occasionally as short, Gram-positive, branching filaments. Filaments were more often seen in the more extensive lesions which tended to occur in the rabbits (Fig. 3). Splenic lesions were curious: Malpighian bodies were surrounded by histiocytes, themselves bordered by an area of fibrinoid necrosis containing polymorphonuclear neutrophil leucocytes but few micro-organisms.

FIG. 2. *A nocardial lesion in the tela choroidea of a rabbit (haematoxylin and eosin × 30).*

FIG. 3. *Gram-positive filaments in the rabbit kidney (Gram stain × 400).*

Intraperitoneal infection of guinea-pigs produced a similar result to that obtained by the intracardiac route. Lesions were, however, more abundant in the liver, mainly in portal tracts.

Intradermal infections remained localized in all but one animal where a few small lesions were ultimately found in the spleen when the animals were killed some weeks later. Prednisone had no effect on the progress of the disease. Indeed the animal showing splenic lesions after intradermal injection had not received the drug.

DISCUSSION

In the absence of pulmonary lesions in nocardiosis other portals of entry may be the gastrointestinal tract and superficial wounds (Ballenger and Goldring, 1957). There was no evidence of oral lesions in the two cases described in this paper and the intestinal tract of Case 1 was normal at necropsy. *Nocardia* are common soil organisms (Gordon and Hagan, 1936) and may cause localized infection in damaged tissues (Abbott, 1956); it is of interest that the appearance of the abscess in Case 2 was preceded by a fall. In Case 1 the patient was a hedger and had ample opportunities to acquire skin abrasions although no history of this was obtained.

Septicaemia following the development of localized abscesses is well recognized and *Nocardia* have been isolated from the blood (Larsen, Diamond, and Collins, 1959). In Case 1 it seems likely that *Nocardia* were disseminated throughout the blood stream about two months before death, being localized in the brain. Subsequently an endocarditis developed with metastatic abscesses in the kidneys. Bacteriological studies made after death were unhelpful; *Nocardia* was not isolated and only coliform bacilli and faecal streptococci were grown from a cardiac valve. The finding of Gram-positive filaments in material from the parasagittal abscesses and failure of cultures of this material to grow *Nocardia* suggests that this infection had been almost eradicated at the time of death.

Localization of the infection, with abscesses, in the brain has frequently been reported (Kaufman and Prieto, 1952; Kirby and McNaught, 1946; Larsen *et al.*, 1959). The histological findings in nocardiosis are those of a non-specific chronic purulent inflammatory process. Endocarditis due to *Nocardia asteroides* has been reported by Cruz

and Clancy (1952); they grew the organism from a heart valve and demonstrated rather atypical filaments in sections. The nature of the endocarditis in Case 1 remains uncertain.

The increase in fungal infections in Great Britain (Riddell, 1956) associated with the use of cytotoxic drugs and steroids may lead to more frequent recognition of nocardial infections. Larsen *et al.* (1959) reported seven cases of nocardial infection; five cases were of leukaemia or malignant disease and three were treated with steroids. The degree of anaemia was not reported but the authors comment on the possible predisposing effect of steroids and cytotoxic drugs.

Although animal experiments performed by us do not support this view with respect to steroids, it seems likely that in patients already anaemic such therapy could enable the infection to become established more easily.

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A family study of the biochemical defects in Wilson's disease

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SYNOPSIS Estimations of serum copper, serum ceruloplasmin (immunochemical), and urinary amino-acids excretion (quantitative and chromatographic) in 44 healthy relatives of patients with Wilson's disease (39 from one family) are reported. Each technique revealed some abnormal individuals. Good agreement was obtained between the serum copper and serum ceruloplasmin estimations and between the quantitative and chromatographic estimations of amino-acid excretion. Some individuals were abnormal to one or other of the pairs of tests only.

These results cast doubt on the hypothesis that the symptoms of Wilson's disease are secondary to a quantitative (or qualitative) abnormality of ceruloplasmin. They also suggest that the mode of inheritance of the biochemical defects may be more complicated than that of a simple recessive mutant gene.

Two of the relatives (one pregnant and one immediately post-partum) had a high serum copper level, as is expected in pregnancy, but normal serum ceruloplasmin. This suggests that the mechanism of control of the serum ceruloplasmin concentration may, normally, depend on the serum copper concentration.

It has been suggested that the symptoms of Wilson's disease may result from deposition of copper in the tissues caused by the deficiency of ceruloplasmin which is thought to be familial and to be transmitted as a recessive characteristic (Scheinberg and Gitlin, 1952; Bearn, 1953; Scheinberg, 1956). In several other familial disorders transmitted in a similar way, *e.g.*, thalassaemia, symptomless biochemical abnormalities have been found in relatives who have therefore been assumed to be heterozygous for the mutant gene.

The only comprehensive family study of the relatives of patients with Wilson's disease was concerned with serum copper and urinary copper excretion (Neale and Fischer-Williams, 1958) although other abnormalities have been reported in individual symptomless relatives, *e.g.*, aminoaciduria (Uzman and Hood, 1952) and ceruloplasmin deficiency (Grouchy, 1958). Neale and Fischer-Williams found abnormally low serum copper levels in a number of symptomless relatives, although the urinary copper excretion was normal. The low serum copper level in these subjects was assumed to reflect a deficiency of ceruloplasmin in symptomless heterozygotes.

One of the manifestations of Wilson's disease, which is attributed to deposition of copper in the renal tubules, is the aminoaciduria. In most typical cases all the biochemical abnormalities are apparent and it is difficult to determine which came first. We now report the results of a study of ceruloplasmin in asymptomatic relatives by an immunochemical technique to confirm the reported deficiency, and of amino-acid excretion to determine whether the aminoaciduria only occcurs in individuals with abnormal ceruloplasmin or whether it also occurs independently.

MATERIALS AND METHODS

Forty-four of the 48 relatives reported by Neale and Fischer-Williams were studied. The same specimens of serum and urine were used as in the previous report. Toluene was added to the urine specimens at the time of

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collection, and the serum and urine specimens were stored frozen at −25°C. The subjects included 39 relatives from one family (family A) all of whom were parents, aunts, uncles, or first cousins (23 on the paternal side and 16 on the maternal side) of two siblings with typical Wilson's disease. The other five were four parents and one aunt of patients with typical Wilson's disease. All the subjects were examined by one of us. None showed clinical evidence of hepatolenticular degeneration. The few coexistent abnormalities were noted in the previous report.

The method and results of serum copper estimation were given in the previous paper (Neale and Fischer-Williams, 1958), and the figures are included in Table I for comparison (normal ranges—male 76 to 133 μg./100 ml.; female 70 to 140 μg./100 ml.).

Serum ceruloplasmin was estimated immunochemically by the method of Gell (1957). Results are expressed as a percentage of a standard serum from a healthy subject, which was stored frozen in divided portions. The method is one of titration, and has an error proportional to the concentration of the unknown (coefficient of variation 15%). The ranges for 47 healthy males were 66-200% (mean 115%) and for 48 healthy females 50-200% (mean 118%). Of these, however, only one female was below 100% (50%) and a repeat estimation on the same individual was 75%. It is doubtful if she can be regarded as normal and she may be heterozygous for the Wilson's gene. Ninety-eight per cent. of healthy females fell between 100 and 200%, which we regard as the normal female range. The range of 10 estimations in four patients with hepatolenticular degeneration was 8-50% (mean 25%).

Urinary free amino-acid excretion was estimated on aliquots of urine collected over 24 hours by the method of Rubinstein and Pryce (1959). The normal range of this method is 75-200 mg. α amino N/24 hours.

Urinary amino-acid chromatography was performed on 46 × 57 cm. Whatman No. 4 paper, using phenol: water and butanol : acetic acid : water (50 : 10 : 40) solvents (Dent, 1948). The chromatograms were developed by dipping the papers in 0·2% ninhydrin in acetone. Standard quantities of 5, 10, 20 and 40 μg. taurine were run on every paper and other amino-acids were compared visually with these spots. Creatinine estimations were done on all the urine samples to confirm the order of accuracy of the 24-hour collection. One ten thousandth of the 24-hour urine volume was applied to each paper to allow comparison of urine amino-acid concentration in different samples. The chromatograms were divided into four grades as follows:—

0 indicates a normal pattern

1 indicates slight excess of amino-acids normally present

2 indicates definite excess of normal amino-acids with small quantities of abnormal amino-acids

3 indicates gross aminoaciduria

The criteria of the normal pattern of amino-acid excretion have been discussed by Dent (1948). Grades 0 and 1 would be regarded as within this normal range, grades 2 and 3 as abnormal. Clearly the urine concentration and the volume placed upon the paper define the final pattern observed and a constant fraction of the 24-hour volume provide a means of comparison between different subjects. Nevertheless, the assessment of the pattern is at best only semiquantitative and the chromatograms classified as grade 2 cannot be regarded as necessarily pathological since the range of normal may be considerable (Berry, 1953). The detailed pattern of amino-acid excretion is shown for each subject (Table II), and it will be observed that leucine, valine, arginine, and lysine occur frequently in the group 2 classification. These amino-acids are not usually seen in the urine chromatograms of normal subjects.

RESULTS

Table I gives the results of these estimations. The serum ceruloplasmin estimations are in good general agreement with the previously reported serum copper results (Fig. 1) except for two sera, one from a pregnant and one from an immediately post-partum woman. The two individuals with serum ceruloplasmin less than the lower limit for the healthy subjects were No. 5 and No. 29, the patient's parents in family A. They also had two of the lowest serum copper levels, There are a considerable number of individuals in the lower part of the normal range who also have a low or low-normal serum copper

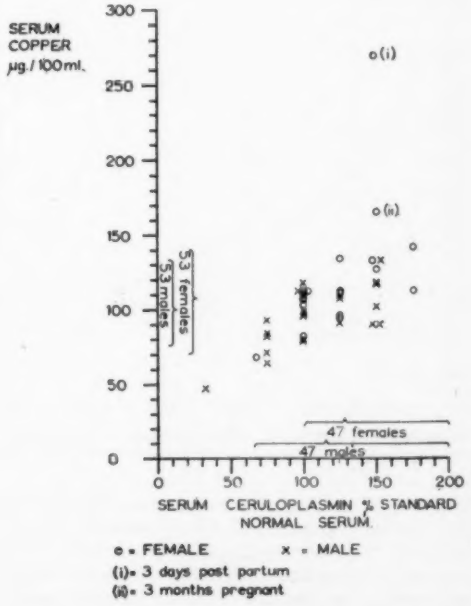

FIG. 1. *The relationship between serum copper and ceruloplasmin in asymptomatic relatives of patients with Wilson's disease. Ranges for healthy adults are given for the two sexes on each axis.*

TABLE I

ESTIMATIONS OF SERUM COPPER, CERULOPLASMIN, AND AMINOACIDURIA IN 44 HEALTHY RELATIVES OF PATIENTS WITH WILSON'S DISEASE

Subject Number	Sex	Age	Relationship to Patient[1]	Serum Copper (μg./100 ml.)	Serum Ceruloplasmin (% of Normal Serum)	Urinary Amino-acid Excretion (mg./24 hr.)	Semiquantitative Chromatographic Grading of Urinary Amino-acids
Paternal Relatives in Family A							
1	M	58	U	117	150	—	0
2	F	56	A	113	100	100	0
3	F	54	A	*82*[2]	100	117	0
4	F	50	A	110	100	126	2
5	M	48	F	*47*	*33*	—	*3*
6	M	45	U	100	150	33	0
7	M	37	C	97	100	—	0
8 (i)	F	29	C	269	150	148	2
9	M	30	C	126	—	190	1
10	F	34	C	113	175	—	*3*
11	F	28	C	96	125	101	0
12	F	27	C	95	125	108	0
13	F	25	C	133	150	153	2
14	M	33	C	91	125	92	2
15 (ii)	F	31	C	165	150	—	1
16	M	26	C	*71*	*75*	82	0
17	M	23	C	*64*	*75*	89	0
18	M	18	C	99	100	78	2
19	M	12	C	112	100	150	0
20	M	19	C	96	100	150	2
21	M	13	C	*84*	*75*	44	1
22	F	9	C	103	100	157	0
23	M	16	C	*82*	*75*	157	1
Maternal Relatives in Family A							
24	F	57	A	109	100	53	0
25	F	53	A	142	175	18	1
26	M	48	U	118	100	190	1
27	F	44	A	112	125	69	0
28	M	42	U	98	—	124	2
29	F	41	M	*68*	*66*	183	2
30	M	38	U	109	125	99	0
31	M	27	C	118	150	96	2
32	F	25	C	107	100	*225*	*3*
33	M	30	C	132	150	*219*	2
34	M	27	C	*80*	100	137	*3*
35	M	22	C	111	—	*270*	*3*
36	F	20	C	112	100	66	1
37	M	15	C	102	150	*225*	2
38	M	15	C	*79*	100	141	2
39	M	12	C	108	125	115	2
Family B							
40	F	61	M	111	125	73	0
41	M	63	F	90	150	78	0
42	F	68	A	127	150	63	1
Family C							
43	F	40	M	134	125	—	—
44	M	42	F	93	*75*	—	—

[1]F = father, M = mother, U = uncle, A = aunt, C = first cousin. (i) = three days post-partum, (ii) = three months' pregnant.
[2]Results regarded as abnormal by the criteria indicated in Fig. 3 are printed in italics.

level. Nine individuals have serum copper less than 85 g./100 ml. (Neale and Fischer-Williams's criterion of low or low-normal). Seven have a serum ceruloplasmin level less than 75% and only one of these is not included in the nine with low-normal copper (the patient's father in family C). The problem in interpreting these data, as a means of dividing these individuals into two populations, normal and abnormal, has already been discussed (Neale and Fischer-Williams, 1958). The ceruloplasmin results only help to confirm that certain individuals are abnormal and that certain individuals are normal for this parameter.

Fig. 2 shows that the two methods of studying amino-acid excretion give general agreement. Several relatives show abnormal aminoaciduria including both parents in family A. The amino-acid pattern (Table II) is similar to that described in overt cases

TABLE II

AMINO-ACIDS DETECTED BY PAPER CHROMATOGRAPHY IN URINE OF 42 HEALTHY RELATIVES OF PATIENTS WITH WILSON'S DISEASE

Subject Number	*24-Hour Volume (ml.)*	*Total α-amino Nitrogen (mg./day)*	*Creatinine (mg./day)*	*Cysteine*	*Aspartic Acid*	*Serine*	*Glycine*	*Taurine*	*Glutamic Acid*	*Threonine*	*Alanine*	*Glutamine*	*Lysine*	*Tyrosine*	*Methionine*	*Histidine*	*Valine*	*Leucine*	*Proline*	*Hydroxy Proline*	*Tryptophane*	*Phenylalanine*	*Methylhistidine*	*β-Hydroxy N. Butyric Acid*	*Arginine*	*Quantitative Grading*
1	620	—	1,220	2	t	2	2		1		1	1		t	t											0
2	1,460	100	1,190	1	t	2	2	t	t	t	t	1														0
3	1,100	117	1,120	1	1	2	2	t			1	1														0
4	1,350	126	1,130	1		1	2	t	t	t	2	1	t	t	t										t	2
5	2,180	—	1,460	4	3	3	3	1	2	2	4	4	1	1	3		t	t		1		t	t	t	2	3
6	1,120	33	810	1	t	2	2			t	2	1												t		0
7	2,380	—	1,450	1	t	2	1	t		t	1	1		t	t											0
8	1,230	148	1,030	t	2	4	3			2	3	3		1	1		1									2
9	1,130	190	1,650	2		3	3	t		1	2	2		1	1										1	1
10	1,500	—	1,380	2	t	2	2		1		1	1		t	t											3
11	1,020	101	1,010	2	t	2	2		1	t	2	1	1	1	1											0
12	1,740	108	1,270	t		3	2	t	1	1	1	1			t										t	0
13	1,460	153	1,140	2	1	3	2	t	2	1	1	2	1	1	1		t	t							1	2
14	1,185	92	1,960	2	1	3	2	t		t	1	2	1	t			t	1							t	2
15	2,230	—	1,470	t	1	2		t	t	t	1	1													t	1
16	1,050	82	1,260	2		2	1			t	t	1														0
17	740	89	1,020	t		2	2			t	t	t		t												0
18	810	78	1,180	2	1	2	2			t	1	1		t				t			t				1	2
19	1,110	150	725	1		2	2				t	t														0
20	1,130	150	870	1	t	2	3		t	t	1	2	t	t	t		t								t	2
21	1,080	44	131	3	t	3	3			2	1	2		t												1
22	1,000	157	780	t	t	2	2			t	1	1		t												0
23	1 410	157	1,240	3	2	4	4			1	2	2	1													1
24	1,940	53	930	t	t	1	2	t		t	1	1														0
25	360	18	284	1	t	3	2	t		t	2	2													t	1
26	1,460	190	1,750	2		2	1	t	1	t	1	1	t	t				t								1
27	1,410	69	1,120	1		3	2		1	t	1	1														0
28	970	124	1,170	t	t			1	1	t	1	1		t	t	t	1	2						t	1	2
29	2,570	183	1,310	2	1	3	3	t	t	t	1	1		t	t									t	1	2
30	900	99	1,360	2	1	2	2	1			2	2	t	t											1	0
31	—	96	—	1	1	3	2	t		t	2	1	t	t	t	1									1	2
32	1,380	225	1,870	t	t	3	2	2	1	2	2	1	1	1	1									1	3	3
33	1,300	219	1,760	2	1	3	3				1	2	1	t	t		t	t							1	2
34	920	137	1,650	2	t	3	3	t		t	2	2		t	t									t	t	3
35	1,890	270	2,240	2	1	3	4	t		1	1	2		t	t								t	t	t	3
36	1,370	66	260	1	t	2	3	t	1	t	t	1													t	1
37	1,060	225	1,150	2	t	2	3	t	2	t	1	1		t				1							t	2
38	1,340	141	1,270	1	t	3	3		2	t	2	2		t				t							2	2
39	1,240	115	460	1	t	3	2			t	2	2	t	t	t		t	t							t	2
40	800	73	750	2	1	2	2			t	1	2											t			0
41	620	78	640	1		2	2	1		t	2	2					t	t								0
42	1,490	63	640	1		1	1		t	t	t	t		t												1

The concentration of each amino-acid is expressed semiquantitatively as trace and grades 1, 2, 3, and 4, and the overall quantitative grading (see text and Table I) is given for comparison.

of hepatolenticular degeneration (Dent, 1948). Here again it is impossible to divide the subjects precisely into two populations, but certain individuals are definitely abnormal and others are normal. For further discussion we regard as abnormal the seven subjects selected on the basis either of having grade 3 chromatograms or more than 200 mg. of total α-amino nitrogen per 24 hours, or both.

There is no evidence of an inverse relationship between the serum ceruloplasmin (and serum copper) and the amino-acid excretion. Some individuals, *e.g.*, subjects 16 and 17, have low ceruloplasmin and copper with normal amino-acid excretion, and others, *e.g.*, subjects 32 and 33, have normal serum ceruloplasmin and copper, but abnormal amino-acid excretion.

A high serum copper level was found in two subjects (8 and 15), who were pregnant or immediately post-partum, as would be expected in normal pregnancy. In both of these, however, serum ceruloplasmin was in the normal (non-pregnant) range.

DISCUSSION

Though our data are of limited value for estimating the incidence of abnormality in family A, certain

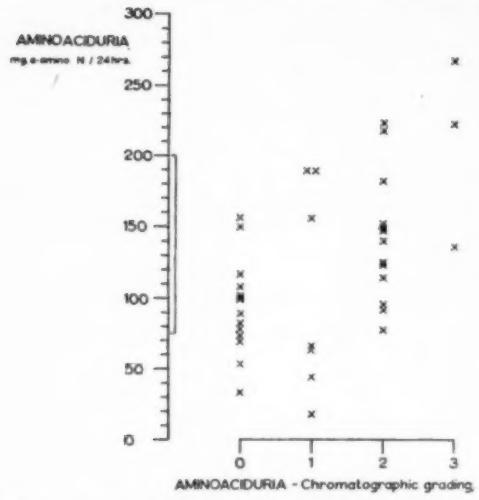

FIG. 2. *The relationship between the chromatographic grading of aminoaciduria and the estimation of the 24-hour amino-acid excretion in asymptomatic relatives of patients with Wilson's disease. The normal range of the latter determination is shown.*

individuals are sufficiently abnormal by each of the tests to demonstrate a familial abnormality which does not give rise to symptoms. Their relationship to the propositi is shown in the family tree (Fig. 3). The estimation of both ceruloplasmin and copper concentrations in parallel helps to define individuals abnormal in this respect. The technique for estimating copper is the more precise, but includes both the non-ceruloplasmin copper, which is raised in Wilson's disease, and the ceruloplasmin copper, which is reduced. The non-ceruloplasmin copper may also be raised in these affected relatives, though our data give no direct evidence. The two parallel techniques of estimating aminoaciduria were also mutually confirmatory. Amino-acid chromatograms of grade 3 are unquestionably abnormal, and, in all but one of such grade 3 urines, the quantitative estimations of α-amino nitrogen were also raised. There is no doubt, moreover, that each of these abnormalities existed independently in certain individuals as well as occurring together.

Other reported studies of such families are somewhat contradictory. They comprise a report of non-quantitated deficiency of serum ceruloplasmin as shown by electrophoresis in starch gel (Grouchy, 1958) and the finding of abnormal urinary amino-acid excretion as shown by paper chromatography and formol titration in five out of six asymptomatic siblings in a family containing four cases of the full disease (Uzman and Hood, 1952). All five were under 13 years old so the symptoms of the disease may still develop, but such an incidence is very surprising if both parents were heterozygous for a single recessive gene. An abnormal urinary peptide was also reported in these subjects; we did not find this in our series. On the other hand, Bearn and Kunkel (1954) failed to find abnormal amino-aciduria in 35 siblings of patients with hepatolenticular degeneration, using a qualitative screening technique. Although the family reported by Uzman

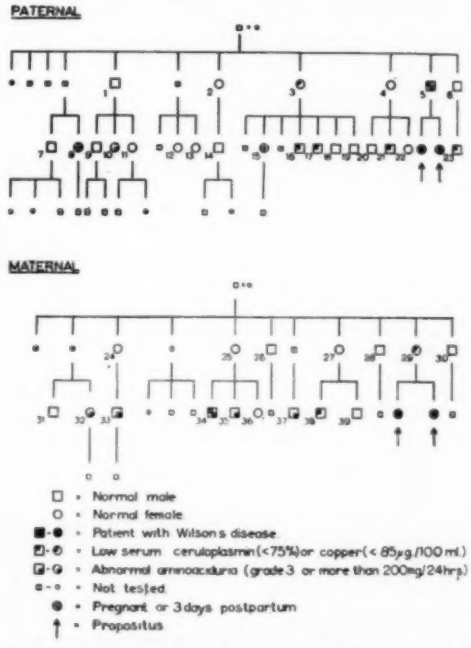

FIG. 3. *Family tree of family A (paternal and maternal relatives of the two propositi). The criteria for defining each individual as normal or abnormal are arbitrary and are discussed in the text. Each one may be assessed in the light of the results given in Table I.*

and Hood (1952) and ours could both have had a symptomless familial aminoaciduria independent of Wilson's disease, this seems an unlikely coincidence. There have been no reports of studies of ceruloplasmin and aminoaciduria in the same family.

A genetically controlled deficiency of ceruloplasmin has often been regarded as the basic abnormality in Wilson's disease (Scheinberg and Gitlin,

1952; Bearn, 1953; Scheinberg, 1956). It has been suggested that this results in an excess of free copper in the blood, which is deposited in brain, liver, kidneys, etc., producing the characteristic morbid anatomical and functional abnormalities, including the aminoaciduria. Three clinically typical cases of Wilson's disease have recently been reported (Sass-Kortsak, Cherniak, Geiger, and Slater, 1959; Rosenoer and Franglen, 1959) in which the serum ceruloplasmin was quantitatively normal but abnormal in its property of combining with copper. These patients had the abnormally increased copper excretion, which is characteristic of the disease, and abnormal deposition of copper in the tissues.

The detection in the same healthy relatives of patients with Wilson's disease of abnormal amino-aciduria but normal serum copper and ceruloplasmin and normal copper excretion make it unlikely that the aminoaciduria is caused by copper deposition in the kidney, whether this is due to quantitative or qualitative abnormality of the ceruloplasmin. The individuals with abnormal serum ceruloplasmin or copper but normal amino-acid excretion are in keeping with previous reports of normal amino-acid excretion in patients with the overt disease (Stein, Bearn, and Moore, 1954; Bickel, Neale, and Hall, 1957). This makes the alternative theory (Uzman and Hood, 1952), that the amino-aciduria is the cause of the disturbance of copper metabolism, untenable. It is, of course, possible that some of the younger of these relatives may still develop the disease but this is unlikely in the case of the two parents (Nos. 5 and 29) who were over 40 years old.

The attraction of the ceruloplasmin deficiency-copper deposition-tissue damage hypothesis of this disease lies in its simplicity, and in its ability to explain all the scattered abnormalities. But there is no precise correlation between the extent of the ceruloplasmin-copper abnormality and the extent of tissue damage and of symptoms. At first the improvement of some patients when treated with copper-removing drugs, such as dimercaprol and penicillamine, appeared to confirm this hypothesis, but recently doubt has been cast on whether their effect depends on their copper-removing action (Walshe, 1960). If, as our results suggest, the renal abnormality is independent of the ceruloplasmin-copper abnormality the hypothesis becomes less attractive. It is equally possible that the cirrhosis of the liver is related to the basic hepatic abnormality of ceruloplasmin production rather than to the copper deposition. There may be a genetically determined deficiency of a widespread tissue enzyme, the deficiency of which makes the liver unable to produce ceruloplasmin and causes cirrhosis; in the brain, it produces mental defect, tremor, etc., and in the kidney aminoaciduria. The generalized copper deposition may be a result of the liver defect and not a cause. Other modifying genes could presumably modify the manifestation of the mutant gene in each one of these situations independently, thus explaining the great variation in the relative severity of the various clinical features in different patients, and the independence of the aminoaciduria and the ceruloplasmin-copper abnormality in our symptomless relatives.

Our data are of limited value for detailed genetic analysis because the limits of normality of the tests applied are difficult to define (particularly for the aminoaciduria) and two of them are only semi-quantitative, so that a statistical comparison of the relatives with the normal population is difficult. Some observations of genetic interest may be made, however. First, the parents of the propositi in family A have much the lowest serum ceruloplasmin and copper levels of all the symptomless relatives. In the theory of inheritance as a simple recessive characteristic, there is no reason why they should differ from any other individuals in the family who are heterozygous for the gene. This suggests that the system is more complex, raising the possibility of modifying genes. Second, the detection of only one doubtfully abnormal test (serum ceruloplasmin 75% in the father of family C) in the four parents of the propositi in the other families strongly suggests a difference between family A and the other two families. (There is no reason why a clinical syndrome may not be inherited in different ways, in different families.)

The discrepancy of copper and ceruloplasmin estimations in the two pregnant (or post-partum) subjects suggests that the disturbance of copper metabolism, which normally occurs in pregnancy (Markowitz, Gubler, Mahoney, Cartwright, and Wintrobe, 1955), may have revealed a latent defect in these individuals. Both showed the high serum copper level (269 and 165 μg./100 ml.) expected in pregnancy, but the serum ceruloplasmin was within normal limits (150%). This is in contrast to our findings in 11 other pregnant women, all of whom had serum ceruloplasmin levels between 200 and 400% of standard 'normal' serum. These high levels in normal pregnancy were commensurate with the high serum copper, so that they continued the linear relationship shown by the other subjects in Fig. 1 and are in keeping with other reports (Markowitz *et al.*, 1955; Scheinberg, 1956). From this discrepancy one can speculate that in pregnancy the raised serum ceruloplasmin normally occurs as a direct result of an increase in serum copper and that these individuals, presumably heterozygous for the mutant gene,

because of their defect, are incapable of fully compensating by producing increased ceruloplasmin. This implies that there is a mechanism of physiological control of the serum ceruloplasmin production, depending on the serum copper concentration. One other piece of circumstantial evidence for this hypothesis is that the serum ceruloplasmin is raised in cirrhosis associated with biliary obstruction (Bearn and Kunkel, 1954). Copper is excreted in the bile, so the elevation of ceruloplasmin in biliary obstruction could be secondary to copper retention.

It has already been suggested (Scheinberg and Morell, 1957) that release of copper from ceruloplasmin in the blood of the alimentary tract, possibly as a result of a low oxidation potential, prevents excessive copper absorption. If both these hypotheses were correct such a mechanism of reciprocal dependance would normally be expected to produce a very precise equilibrium, such as is indeed found in the remarkably constant serum copper concentration.

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A study of six representative methods of plasma bilirubin analysis

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SYNOPSIS The large number of modern methods which have been published for the determination of total bile pigments in the blood plasma indicates the difficulties of the estimation and suggests the need for a reliable standard method. In view of the present importance of plasma bilirubin levels in the diagnosis and treatment of jaundiced subjects, six analytical procedures have been studied, each chosen as far as possible, as a representative of a group of methods each embodying different principles.

A spectrophotometric method (White, Haidar, and Reinhold, 1958) and five diazo coupling procedures (Jendrassik and Gróf, 1938; Powell, 1944; Perryman, Richards, and Holbrook, 1957; Stoner and Weisberg, 1957; Lathe and Ruthven, 1958) were investigated, and the results obtained by carrying out duplicate analyses by different methods on 54 stored and 64 fresh plasmas or sera are presented and discussed in this paper.

An amount of haem pigment which can be detected visually in plasma is sufficient to cause a considerable underestimation of bilirubin by the method of Powell (1944) and overestimation by the method of Stoner and Weisberg (1957). Inferences drawn from serial estimations by these methods can therefore be misleading, and this is especially pertinent when results obtained from infant blood containing variable amounts of haem pigments are being assessed. Methods based on the original van den Bergh procedure with various preliminary steps of protein precipitation in alcoholic solutions at about *p*H4 give low and poorly reproducible results; losses due to the co-precipitation of bilirubin esters with protein have been confirmed. In our opinion such methods should be abandoned.

Determination of total bilirubin by colour (White *et al.*, 1958) is reliable only for plasma known to contain no lipochrome or 'directly-reacting' pigment; total bile pigment may be overestimated by this technique in plasma containing conjugated bilirubin.

We consider that the method of Lathe and Ruthven (1958) is to be preferred for use on a routine basis. It is conveniently simple, and under all conditions normally encountered gives results sufficiently accurate and reproducible for clinical use. When this or any other procedure is used, a primary standardization between different laboratories with sera containing known amounts of added bilirubin is essential if comparable results are to be obtained.

There is no reliable correlation between the concentration of bile pigments in the plasma and the intensity of clinical jaundice (With, 1954; Culley, Waterhouse, and Wood, 1960). Both the free and the conjugated forms of bilirubin are bound to plasma albumin which, because it pervades cutaneous and other tissues, can cause clinical jaundice. This occurs in adults (with obstructive jaundice) at levels of between 2 and 4 mg. bile pigment per 100 ml. plasma. In infants with or without haemolytic disease, the cellular diffusion of the protein-bound pigment appears to be hindered, and a plasma level of 5 to 9 mg. bilirubin per 100 ml. is often required to produce jaundice.

Serum or plasma bilirubin assays are therefore used to assess the progress of jaundiced subjects, and also to detect small increases of pigment such as may occur in the plasma of cases of early hepatitis, in carriers of homologous serum hepatitis, in some macrocytic anaemias, and in patients receiving treatment with various drugs. A knowledge of bilirubin

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levels is important at the present time for directing the treatment of jaundiced newborn babies, and, partly for this reason, many new micro methods for determining bilirubin levels have recently been devised.

With (1954) reviewed the methods available up to the end of 1951, but since that time no less than 23 new or adaptive procedures have been reported. Although a few of these publications include a mention of a small number of analytical results obtained by the proposed and the parent method, usually that of Malloy and Evelyn (1937) or King and Coxon (1950), no substantial comparative studies appear to have been made.

The work now being reported was undertaken to find out whether appreciable or significant differences in the results of bilirubin analyses exist between different methods, and also to assess the extent and to discover the cause of such variations when present.

THE METHODS

The methods employed were as follows.

1 The method of Powell (1944) for plasma containing up to about 5 mg. bilirubin % was used as published. In this method, the conventional diazo reagent is added to the plasma, followed by an excess of sodium benzoate-urea solution. A plasma blank test is carried out in which dilute HCl replaces the diazo reagent. Colour densities were read in an EEL colorimeter with Ilford filter 625. Analyses on the more deeply pigmented plasma were made with 0·5 ml. of 1 in 5 saline dilutions of plasma. This method was chosen as a representative of the class employing a non-alcoholic coupling promoter. It was the method routinely used in this hospital at the beginning of our study.

2 The method of Jendrassik and Gróf (1938) was used as published; this is also a non-alcoholic coupling procedure. In it, a solution containing sodium acetate, caffeine, and sodium benzoate is added to plasma followed by a diazo reagent prepared by mixing 0·25 ml. nitrite solution with 10 ml. of 0·5% sulphanilic acid in 0·15 N-HCl. Upon the addition of excess alkali, the pink azo pigments are converted to blue salts. A plasma blank was carried out. The reagent blank was zero. The extinction of the final green solution was read in an EEL colorimeter using an Ilford 607 filter. This was the only departure made from the original method of Jendrassik and Gróf who employed a Pulfrich photometer with filter S61. The method has been re-published in English by Fog (1958a).

3 The method of King and Coxon (1950) modified in a manner suggested by Perryman *et al.* (1957) was used. Ammonium sulphamate (0·15%) is incorporated in the diazo reagent and a few crystals of sodium azide are added after the addition of ethanol (85% v/v). Readings are made at 530 mμ and 425 mμ, and the azo pigment extinction is then corrected for interference by haem pigments by means of a simple formula.

As no photomultiplier was available, we used 0·2 ml. plasma for every test, giving a final plasma dilution of 1 in 50 compared with 1 in 100 stipulated by Perryman *et al.*, and 1 in 10 as used in the original procedure by King and Coxon. The initial plasma dilution was made with water. Optical densities were read on a Unicam SP 600 photoelectric spectrophotometer. This method was chosen as a representative of the class of methods involving deproteinization before diazo coupling.

4 Rice (1957) has suggested that the method of Stoner and Weisberg (1957) for plasma bilirubin estimation may 'fulfil reasonably well the criteria of an ideal analytical procedure'. This technique also involves deproteinization, but uses the measurement of acid blue azo pigments as an end-point device. It was on this latter account chosen for study. Regrettably, it was not found possible to employ the technique as published, because we were unable to obtain a cuvette of 1 cm. stratum length suitable for measuring optical densities with only 1·7 ml. solution. The method had therefore to be scaled up. Care was taken to keep the concentration of the reagents as nearly as possible identical to those of the original procedure. We used 0·25 ml. diazo reagent and 0·1 ml. plasma for all tests. Coupling was allowed to proceed in a constricted calibrated centrifuge tube for 10 min., after which 0·3 ml. 10 N-HCl and 0·5 ml. aqueous ammonium sulphate (saturated at the time of use) were added. The volume of solution was made up to 5·0 ml. with ethyl alcohol (absolute), the mixture shaken vigorously for 1 min., and then allowed to lie almost horizontally for 15 min. After filtration through Whatman No. 40 paper the optical density of the supernatant fluid was measured at 580 mμ against a reagent blank. No serum or plasma blank is used with this test.

5 The method of Lathe and Ruthven (1958) was used as published. The diazo reagent consists of a mixture of 0·3 ml. of sodium nitrite (0·5%) and 10 ml. of 1% sulphanilic acid in 0·2 N-HCl. Coupling takes place in 50% methanolic solution. A plasma blank is performed in which 1% sulphanilic acid in 0·2 N-HCl replaces the diazo reagent. The azo pigment colour usually reaches its maximum intensity about 5 min. after the alcohol has been added, but with some grossly pigmented specimens up to 9 min. are required. Tests were routinely read at 10 min. in an EEL colorimeter with an Ilford 624 filter (525 mμ). The method was chosen as a representative of the class of non-precipitation methods employing an alcohol as the coupling promoter.

6 Direct spectrophotometric assays were made in duplicate on fresh samples of heparinized plasma by the method of White *et al.* (1958). We used, for all tests, 1 in 50 dilutions of plasma in M/15 phosphate buffer (1·74 g. KH_2PO_4 and 14·4 g. $Na_2HPO_4\,7H_2O$ per litre), *p*H 7·1. Extinctions were read at 455 and 575 mμ in a Unicam SP 600 with 1 cm. matched cells.

STANDARDIZATION OF METHODS The original method of Powell is calibrated from one bilirubin solution (4 mg. %), but, as usually employed, it is standardized from a density/concentration curve obtained by testing serial dilutions of a chloroform-alcohol solution of bilirubin in place of serum (Varley, 1958). We adopted the former

procedure for specimens containing less than about 4 mg. % bilirubin, and the latter for all other tests. In either case, alcohol replaces benzoate-urea as a coupling promoter. The method of Lathe and Ruthven is also standardized from one solution prepared by dissolving a known amount of bilirubin in chloroform. This is then diluted with alcohol and treated in a similar manner to that of the test sample. The method of Perryman *et al.* employs a buffered methyl red solution as a standard for everyday use. The method of Lathe and Ruthven includes a check on colorimeter responsiveness with the aid of a solution having a constant optical density, *i.e.*, Thompson's neutral grey solution. With the exception of the Stoner and Weisberg and the Jendrassik and Gróf results, our plasma bilirubin results were calculated by reference to the standards advised by the authors of the respective methods. All results were also calculated by reference to standard graphs obtained by the procedures now to be described.

One of us donated blood from which 200 ml. of sterile clear yellow serum was obtained. This was used as a medium for stabilizing bilirubin in solution. Our earlier attempts to use discarded transfusion plasma for this purpose were unsuccessful because sodium citrate interfered with some methods of analysis, and a loss of bilirubin was experienced following its addition to glucose-citrated plasma which had been stored for three weeks at 4°C.

The fresh serum was exposed to ultra-violet light for three periods of two hours on each of three successive days, at the end of which the bilirubin concentration had fallen from its original level of 0·7 mg. to one of less than 0·2 mg. per 100 ml. Forty milligrams bilirubin were rapidly dissolved by stirring in 2·0 ml. 0·100 N-NaOH, immediately diluted with about 80 ml. serum, mixed with 2·0 ml. 0·100 N-HCl, and finally more serum added to make a volume of 100 ml. Serial dilutions from this stock solution were made with the original serum diluted with 4 ml. 0·1 M-NaCl per 100 ml. The standard sera thus prepared contained 2, 5, 10, 15, 20, 25, 30, and 40 mg. bilirubin per 100 ml. Using 0·1 ml. of these standards, the methods of Powell and Jendrassik and of Gróf were applied. The range between 6 and 35 mg./100 ml. was later used for the calculation of the patient's plasma bilirubin. With the method of Lathe and Ruthven, 0·1 ml. was used for the range 4 to 16 mg./100 ml., and 0·05 ml. for levels of 15 to 35 mg./100 ml. Of the same standard sera 1 in 50 dilutions were employed to standardize the methods of Stoner and Weisberg, Perryman *et al.*, and White *et al.* for the complete range of bilirubin concentrations encountered. Before use, the wavelength scale of the Unicam SP 600 was calibrated against a didymium filter according to the makers' instructions. The wavelength scale was considered to be true to $\pm 0{\cdot}5$ mμ over the range 420 to 620 mμ.

A further series of standard sera containing 0·5, 1·0, 1·5, 2·0, 3·0, 5·0, 6·0, and 8·0 mg. bilirubin/100 ml. were similarly prepared from a stock serum containing 10 mg. bilirubin/100 ml., and the analyses carried out on these specimens using 0·4 ml. (by the methods of Jendrassik and Gróf and Lathe and Ruthven) and 0·5 ml. (by the method of Powell). Blank tests were made on the same volume of the corresponding standard bilirubin serum.

All the standardization procedures were completed within a period of three days. The standard solutions (and subsequently the test plasmas) were analysed at various laboratory temperatures between 17·5 and 19·5°C. Eastman-Kodak bilirubin (batch No. 23A) with a millimolar extinction coefficient of 59·3 in aldehyde- and phosgene-free chloroform was used throughout.

THE SPECIMENS FOR ANALYSIS Heparinized blood plasma (10 to 20 i.u. of dry lithium heparin per ml.) was obtained from each of two normal healthy women, nine adults with various liver diseases, and 30 jaundiced infants aged 1 to 7 days. Serum was obtained from the cord blood of each of 13 infants. The specimens were stored at −10°C. in the dark until the day before the start of the analyses, when they were transferred to a refrigerator (5°C.). Twenty-four specimens containing 0·2 to 4 mg. % bilirubin were allowed to warm to room temperature, and then analysed by each of the diazo methods within a period of four days. When not in use, the specimens were stored at 5°C. in the dark. On the last of the four days, some analyses were repeated to check any loss of bilirubin which might have occurred. Sixteen specimens containing from 4 to 15 mg. % and 15 specimens containing from 15 to 35 mg. % bilirubin were then analysed under similar programmes.

A further series of analyses for pigments was carried out on 64 fresh specimens of heparinized plasma. Tests were made by a chosen diazo-coupling technique and by the spectrophotometric method of White *et al.* (1958); 'directly-reacting' bilirubin was also estimated by the method of Lathe and Ruthven (1958). Forty-four specimens were obtained from 15 premature babies, eight of whom were jaundiced, and from four infants with erythroblastosis neonatorum, one of whom developed the inspissated bile syndrome. In addition there were six specimens derived from the umbilical cord vein at parturition, and 14 specimens from adult female patients, including cases of toxic hepatocellular disease or obstruction of the common bile duct.

RESULTS

We were unable to obtain reproducible results by the method of Perryman *et al.* Varying amounts of unconjugated and conjugated bilirubin were demonstrated by extraction and subsequent diazo coupling to be retained on the precipitate, and the extent of retention was influenced by the amount of shaking given to the mixtures. This variable loss of pigment was also observed when the analyses were carried out as soon as possible after the collection of blood samples from infants with haemolytic jaundice. An examination of the individual results obtained in the duplicate analyses by the other four methods revealed no obvious difference in the reproducibility of the methods. With two exceptions these individual figures were within 3% of the mean value quoted.

The final coloured solutions from the following

specimens were opalescent:—Nos. 1, 41, 54 (method of Lathe and Ruthven); Nos. 1, 2, 41, 53, 54 (method of Powell); Nos. 1, 2, 41, 54 (method of Jendrassik and Gróf).

The mean results by the methods of Perryman *et al.*, Powell, and Lathe and Ruthven when calculated from standardizations against bilirubin-containing sera are at variance with those calculated as recom-

TABLE I

PLASMA BILIRUBIN LEVELS (MG./100 ML.) OBTAINED BY DIFFERENT METHODS

Sample	*Perryman* et al. *(1957)*		*Stoner and Weisberg (1957)*	*Powell (1944)*		*Jendrassik and Gróf (1938)*	*Lathe and Ruthven (1955)*	
	(a)[1]	*(b)*[1]	*(b)*	*(a)*	*(b)*	*(b)*	*(a)*	*(b)*
1[2]	0·1	0·1	0·4	0·4	0·3	0·2	—	0·3
2[2]	0·3	0·2	0·5	0·4	0·3	0·3	—	0·3
3D	3·4	3·0	4·1	4·4	4·4	3·9	—	4·7
4D	1·9	1·5	2·6	3·1	2·9	2·7	—	3·0
5D	1·9	1·5	4·2	4·0	3·9	3·7	—	4·1
6	1·3	1·0	1·6	1·9	1·8	1·9	—	2·1
7D	2·9	2·4	3·9	3·8	3·7	3·6	—	3·9
8	0·9	0·8	2·2	1·8	1·6	1·5	—	1·7
9D	1·6	1·2	2·0	2·5	2·3	2·1	—	2·4
10DH	1·3	1·0	2·4	2·0	1·8	2·0	—	2·1
11H	1·3	1·0	2·4	2·3	2·1	2·0	—	2·3
12[2]H	1·4	1·2	—	2·2	2·0	1·9	—	2·2
13	1·2	1·0	1·5	1·7	1·5	1·5	—	1·9
14D	2·8	2·3	2·2	2·2	2·0	1·9	—	2·7
15	1·7	1·3	2·6	2·1	1·9	1·9	—	2·3
16H	0·8	0·7	1·5	1·4	1·2	1·4	—	1·7
17DH	2·3	1·9	3·6	3·2	3·0	3·1	—	3·5
18H	0·8	1·2	2·1	1·2	1·1	1·3	—	1·4
19	1·3	1·0	2·0	2·1	1·9	1·9	—	2·3
20D	3·1	2·7	4·4	3·6	3·5	3·4	—	3·9
21	2·2	1·8	2·5	2·5	2·4	2·4	—	2·7
22D	2·5	2·1	4·2	3·6	3·5	3·5	—	4·1
23[2]	0·4	0·4	1·2	0·3	0·3	—	—	—
Mean of values	1·8	1·5	2·7	2·6	2·4	2·4	—	2·8
24D	3·5	3·1	4·8	4·4	4·5	4·2	4·4	4·9
25DH	8·1	6·3	10·5	7·9	8·5	8·7	8·5	9·5
26	6·3	5·3	7·6	7·0	7·4	6·0	6·3	7·1
27	8·9	8·2	10·4	9·2	9·9	9·2	8·8	9·9
28	6·9	6·1	8·2	7·1	7·6	7·3	6·8	7·6
29DG	2·2	2·0	5·3	3·3	3·3	3·3	3·5	4·0
30H	8·6	7·8	11·7	9·6	10·3	9·7	9·1	10·2
31H	10·8	10·0	13·0	10·2	10·9	10·9	8·8	9·9
32H	10·2	9·4	12·4	10·9	11·8	11·5	10·2	11·5
33H	12·8	12·0	13·9	12·1	13·2	13·5	11·6	13·0
34H	11·6	10·7	13·6	12·0	13·1	12·4	12·8	14·2
35H	12·4	11·1	15·1	10·8	11·6	13·7	12·3	13·7
36	10·5	9·6	12·1	10·9	11·8	11·5	10·3	11·5
37	11·6	10·8	12·7	12·4	13·4	12·9	11·9	13·2
38D	13·3	12·6	17·6	12·9	14·2	15·1	12·6	14·3
39	14·7	14·0	13·4	12·6	13·8	13·6	13·7	15·3
40H	14·8	14·1	16·7	14·2	15·7	16·8	15·9	17·8
41DL	17·2	16·6	24·1	16·6	18·8	19·2	18·2	20·2
42H	14·6	13·8	18·0	14·3	16·3	16·3	14·4	16·1
43	14·0	13·2	15·5	14·8	16·4	15·2	14·2	15·9
44	15·9	15·0	18·5	13·6	17·4	16·8	15·7	17·6
45	14·5	13·9	17·2	15·5	17·3	16·8	15·1	16·9
46	18·7	18·0	23·0	20·4	24·0	23·5	21·0	22·7
47H	15·7	14·9	17·6	13·7	15·0	16·2	14·7	15·8
48D	14·0	13·3	15·6	13·9	15·3	15·2	13·6	14·8
49	21·9	21·3	24·5	21·2	25·2	24·0	21·4	22·6
50	29·0	29·4	32·3	26·4	32·7	31·0	28·6	30·8
51	27·2	27·2	32·6	25·8	31·7	30·8	27·4	29·7
52	29·1	29·7	37·0	28·0	35·4	35·0	31·9	34·2
53DL	13·0	12·3	16·6	12·8	14·1	14·2	13·3	14·4
54DHL	12·2	11·4	15·2	11·6	11·4	12·5	9·7	12·6
Mean of values	13·7	13·0	16·3	13·5	15·2	15·1	13·8	15·3
Mean of all values	9·2	8·6	11·2	9·4	10·4	10·3	—	10·5

[1](a) Standardized as recommended by author(s) of method, and [1](b) standardized as described in text.
[2]Not included in the calculation of mean values.
D = containing visually detectable amount of 'direct' pigment.
H = containing > 0·2% haemoglobin.
L = grossly lipaemic specimen.
G = green specimen.

TABLE II

EFFECT OF HAEM PIGMENTS AND BILIRUBIN ESTERS IN PLASMA ON SOME TOTAL BILIRUBIN ESTIMATIONS

	No of Specimens	*Mean % Deviation from Approximate True Values*[1]			
		Perryman et al. *(1957)*	*Stoner and Weisberg (1957)*	*Powell (1944)*	*Jendrassik and Gróf (1938)*
Unhaemolysed plasma					
'Direct' pigment	14	−34	+3	−4	−8
No 'direct' pigment	20	−25	−1	−1	−6
Total	34	−28	+1	−2	−7
Haemolysed plasma					
'Direct' pigment	4	−38	+12	−13	−6
No 'direct' pigment	12	−22	+10	−7	−4
Total	16	−26	+10	−9	−4

[1]Values obtained by the method of Lathe and Ruthven (1958).

mended by the respective authors. Considering the results based on our mode of standardization (Table I), it is apparent that those obtained by the method of Perryman *et al.* are appreciably lower (0 to 60%) than the results found by the other diazo methods. When subjected to a statistical analysis, the results obtained by the remaining methods could be divided into two similar groups: those of Stoner and Weisberg and of Lathe and Ruthven, and those of Powell and of Jendrassik and Gróf. The latter group of values were the lower, and for reasons which will be discussed, it seemed highly probable that the Stoner and Weisberg and the Lathe and Ruthven values were close to the correct results.

Using the values obtained by Lathe and Ruthven's method as a basis, a comparison of all results is shown in Table II, from which it will be seen that bilirubin in haemolysed specimens appears to be overestimated by the method of Stoner and Weisberg, and underestimated by that of Powell. With the method of Perryman *et al.*, total bilirubin is more incompletely estimated in plasma containing 'directly-reacting' pigment than in specimens not containing bilirubin in this form.

During the period of storage, some of the haemoglobin originally present in the haemolysed specimens was converted to other haem pigments. Because this and possibly other protein changes might have affected the analyses, three fresh specimens of heparinized bile-pigmented plasma, each slightly haemolysed, were analysed by the five diazo methods. Each gave results which varied in a similar manner to those obtained on the stored plasma.

Table III summarizes the results obtained for bile pigments on 64 fresh samples of plasma. The specimens from the jaundiced adults and one infant contained large amounts of 'directly-reacting' bilirubin. In these instances the mean pigment

TABLE III

PLASMA BILE PIGMENT LEVELS DETERMINED SPECTROPHOTOMETRICALLY AND BY DIAZO COUPLING

	Total Bilirubin (mg./100 ml.)		*%Bilirubin Reacting 'Directly'*
	White et al. *(1958)*	*Lathe and Ruthven (1958)*	*Lathe and Ruthven (1958)*
Adult plasma (obstructive jaundice)	16·5	15·1	72
	13·8	12·1	72
	11·8	9·2	59
	2·9	1·5	47
Adult plasma (hepatocellular jaundice)	19·1	18·0	65
	8·6	7·8	72
	8·2	7·7	75
	7·6	7·3	66
	5·5	5·7	63
	4·4	3·3	>20
	4·1	3·3	>20
	2·7	2·4	38
	2·4	1·1	>20
	1·8	1·7	>20
Infant plasma (inspissated bile syndrome)	19·9	16·1	71
	10·4	8·3	52
	5·9	5·0	60
	5·6	4·3	61
Mean of above specimens	8·4	7·2	>50
Mean 40 infant plasmas	12·8	13·2	<10
Mean 6 cord plasmas	2·2	2·3	<10

concentration found by spectrophotometric analysis (method of White *et al.*) exceeded that found by diazo coupling (method of Lathe and Ruthven) by from 0 to 93% (mean, 16%).

With the exception of the case of inspissated bile syndrome, all the infant plasma (from capillary and cord blood) contained less than 10% of pigment which reacted 'directly', Total bile pigment ranged from 1·8 to 29·1 mg./100 ml. The mean pigment level found spectrophotometrically in these specimens was 96·5% (S.E. of mean 0·8%) of that obtained by the diazo method.

DISCUSSION

DIAZO METHODS WITH PROTEIN PRECIPITATION The claim of Perryman *et al.* (1957) that absorption of bilirubin esters on the precipitated protein can be prevented by decreasing the serum concentration in the reaction mixture to 1% has not been confirmed by the present work. Several of our stored plasmas were tested in parallel on dilutions of 1 in 20 and 1 in 100, and were found to give almost identical results. Tests on plasma from freshly drawn blood also gave low results, so that it is unlikely that denaturation of protein during the storage period was responsible for the poor results.

The time allotted for the development of the azo-pigment colour in the method of Perryman *et al.* is 5 min. We allowed 10 min. since this was more convenient for batch analyses. An extension of this period to 30 min. (the time employed by King and Coxon) produced no appreciable increase in azo-pigment density, and the omission of the 'few crystals of sodium azide' to destroy excess diazonium compound also had no effect on the final pigment density. In the procedure of Perryman *et al.* the other deviation from that of King and Coxon's method is the addition of 1 ml. of 1·5% ammonium sulphamate to 10·3 ml. of diazo reagent before use. This modification was first suggested by Patterson, Swale, and Maggs (1952) as a means of destroying excess nitrous acid. We were unable to detect excess nitrite in the diazo reagent of King and Coxon, which is not surprising in view of the fact that the reagent contains only 38% of the theoretical nitrite requirement of the sulphanilic acid. The omission of the ammonium sulphamate had no effect on the analysis.

Two comparative studies (Patterson *et al.*, 1952; Lathe and Ruthven, 1958) have previously indicated that the bilirubin values obtained by the method of King and Coxon (1950) are lower than those found by some non-precipitation methods. From the present work it appears that this type of precipitation method is incapable of accurately determining serum or plasma bilirubin. That this has not been generally recognized is partly due to the fact that with King and Coxon's method, non-specific chromogens in the aqueous alcohol extract often contribute to the final colour, thereby improving the apparent bilirubin result.

The methyl red solution of Haslewood and King (1937) employed as a standard by Perryman *et al.* (1957) did not in our hands match the shade of colour or the optical density of the azo pigments derived from the quoted concentration of our bilirubin preparation when the latter was prepared as a standard bilirubin serum. We found (with the method of Perryman *et al.*) very close agreement between the corrected and the uncorrected $^{E}530$ mμ values on each of the standard bilirubin sera, and the bilirubin results obtained for haemolysed and non-haemolysed plasma seem to indicate that the haem-pigment correction formula is adequate (see Table II).

In the case of Stoner and Weisberg's method, acid haematin when present in the final solution absorbs appreciably at 580 mμ, the wavelength at which the blue pigments are measured. This would account for the higher values obtained by this rather than by Lathe and Ruthven's method, since the latter is relatively unaffected by haem pigments (Watson, 1960). We noted that small but variable optical readings could be obtained with blank tests on different plasma containing approximately the same amount of bilirubin, but the matter was not further investigated.

DIAZO METHODS WITHOUT PROTEIN PRECIPITATION The methods of Jendrassik and Gróf (1938) and of Powell (1944) are modifications of a method devised by Enriques and Sivó (1926) in which the coupling promoter is sodium benzoate. Such methods have been criticized on the grounds that the final colours are sometimes 'impure' (King and Coxon, 1950), and that they require several hours for the complete coupling of serum bilirubin in cases of haemolytic jaundice (Gray, 1953; Stoner and Weisberg, 1957). In our experience these criticisms apply to those modifications of Enriques and Sivó's method in which sodium benzoate has been omitted in favour of a less efficient coupling promoter, *e.g.*, caffeine or citrate (Rappaport and Eichhorn, 1943; Gray and Whidborne, 1946; Chabrol, Böszörményi, and Fallot, 1949). During the course of several thousand bilirubin analyses at this hospital by Powell's method, we have never observed 'impure' colours, nor have we been able to detect any further increase in azo pigment density with time. Nevertheless, false low bilirubin values are obtained by Powell's method if the plasma contains traces of

haemoglobin or haem pigments. This effect is caused by interference with coupling, rather than—as was suggested by Sims and Horn (1958)—excessive 'blank' correction. Since with fresh haemolysed plasma (naturally or artificially bile pigmented) we have observed larger underestimates of bilirubin by Powell's method (Watson, 1960) than were actually found with the stored specimens, it is possible that some conversion products occurring in our specimens as a result of storage in deep-freeze interfere to a lesser extent with bilirubin coupling than haemoglobin does.

The final solutions of the Malloy and Evelyn (1937) procedure derived from lipaemic plasma or specimens containing normal concentrations of bilirubin are occasionally opalescent, and it is generally agreed that the intensity of this turbidity may vary between the 'blank' and the 'test' determinations. As previously stated, we observed opalescence with Lathe and Ruthven's method, but the benzoate methods of Powell, and Jendrassik and Gróf were also equally subject to this source of error.

Several methods of bilirubin analyses, including those of Powell (1944), Dangerfield and Finlayson (1953), Sims and Horn (1958), and Brückner (1959), employ insufficient diazo reagent to hydrolyse and couple all the bilirubin in deeply bile-pigmented plasma, unless a smaller volume of plasma than that stipulated is taken for analysis (Watson, 1961). Lathe and Ruthven in their method suggest using half the quantity of plasma for specimens containing more than 15 mg. total pigment per 100 ml. However, there is nothing obligatory or distinctive in the change at 15 mg., since the method employs per millilitre of plasma much higher final concentrations of diazotised and undiazotised sulphanilic acid than is used in the parent method of Malloy and Evelyn (1937). On the other hand, when inadvertently using 0·1 ml. plasma containing more than 20 mg. bilirubin per 100 ml., we have encountered some plasma specimens from which the appearance of maximum azo pigment colour was delayed for 15 to 20 min. Contrary to the contention of Meites and Hogg (1959), we found it an advantage to use different plasma concentrations because it enabled us to read the final optical densities more accurately. For this reason also, we used a volume of 0·4 ml. for plasma which appeared to contain normal or only slightly above normal concentrations of bile pigment.

Meites and Hogg (1959), using the method of Malloy and Evelyn (1937), have shown that when the temperature at which the analysis is performed is dropped from 22°C. to 10°C., the production of azo pigments from serum bilirubin is decreased by about 50%. The density of the azo pigments produced from chloroform solutions of bilirubin under similar conditions, however, does not vary by more than 5%. Since in our laboratory, room temperatures fluctuate from 13°C. to 26°C. during the year, Malloy and Evelyn's method would require careful temperature control, or standardization at different temperatures with bilirubin sera, procedures inconvenient for routine use. A modified reagent which reacts maximally in this temperature range has been proposed, but unfortunately the results obtained with it are very considerably affected by subvisible amounts of haemoglobin in serum (Meites and Hogg, 1959). Using the method of Lathe and Ruthven we have observed that no decrease occurs in the production of azo pigment from plasma containing bilirubin as a result of a fall in the laboratory temperature. Moreover, this method has been shown not to be significantly affected by haemolysis (Watson, 1960).

The method of Jendrassik and Gróf has been stated to have a high degree of specificity and reproducibility (With, 1954; Fog, 1958a). If we assume that the bilirubin levels found by this method are correct, then the results obtained by the method of Stoner and Weisberg, Lathe and Ruthven, and Powell on unhaemolysed specimens will be 5 to 10% too high. The occurrence of side reactions producing yellow and brown diazo products with urobilinogen (Lopez Garcia, 1941) and with some non-protein nitrogen compounds (Harrison and Bromfield, 1928) have been reported. But these reactions cannot here account for an overestimation of pigment by the three methods, since, with the exception of plasma No. 9, none of our specimens was azotaemic, and it is more likely that the infant plasma tested contained lower rather than higher than average concentrations of urobilinogen. Caffeine-benzoate coupling methods have previously been reported to give lower values than those found by some alcohol coupling methods (Malloy and Evelyn, 1937; Ducci and Watson, 1945). It is therefore possible that some substance present in the plasma of jaundiced patients (and absent from normal serum fortified with bilirubin) may inhibit the coupling of bilirubin under the conditions of Jendrassik and Gróf.

Results based on standardizations from chloroform solutions and serum solutions of bilirubin differed by approximately 9% (methods of Powell and of Lathe and Ruthven). In chloroform solutions, the relative extinction of 'azobilirubin' was higher than in serum solutions; the coupling promoter used in each case was 50% methanol. Using 80% ethanol, O'Hagan, Hamilton, Le Breton, and Shaw (1957) observed 2% lower azo pigment densities with choroform bilirubin solutions than with the same quantities of bilirubin in serum (method of Powell). Although there is no doubt that changes in the spectral curves produced by variations in solvent

and *p*H are the cause of part of the discrepancies, we are unable to account satisfactorily for the large differences. Experiments in which bilirubin was added to bile-pigmented plasma have always yielded 100 ± 2% recovery.

SPECTROPHOTOMETRIC METHOD Higher total bile pigment levels for adult plasma were obtained by spectrophotometry by the method of White *et al.* than by diazo coupling by the method of Lathe and Ruthven. This is contrary to the opinion of White *et al.* (1958) but in agreement with the findings of Fog (1958b) who measured pigment colour by a more refined procedure (Fog, 1949). On the other hand, the bile pigment levels obtained by the diazo and spectrophotometric methods showed good agreement in plasma obtained from infants (both icteric and non-icteric) during the first week of life. An exception was the infant with the inspissated bile syndrome (whose plasma bile pigment was mainly in the conjugated form). Since infant plasma is almost devoid of lipochromes (Mertz and West, 1956), it would appear that estimation by the method of White *et al.* (1958) includes in addition to lipochromes when present, a measure of non-bilirubin pigments which are exclusively found in plasma containing an appreciable percentage of bilirubin reacting 'directly' by the method of Lathe and Ruthven. These pigments may be dipyrroles, *e.g.*, bilifuscin or allied pigments as suggested by With (1954), or possibly bilirubin esters or non-diazo coupling tetrapyrroles with a higher specific extinction coefficient than unconjugated bilirubin.

CONCLUSIONS

Haem pigment known to be present in many specimens of infant plasma causes the method of Powell (1944) to underestimate and that of Stoner and Weisberg (1957) to overestimate the plasma bilirubin concentration.

Bilirubin methods incorporating protein precipitation, *e.g.*, that of King and Coxon (1950), and modifications, give low and variable results.

Results obtained by spectrophotometry (White *et al.*, 1958) are satisfactory for infant plasma, provided no appreciable amounts of 'directly-reacting' bilirubin are present.

Experience with several techniques has led us to choose that of Lathe and Ruthven (1958) as the most suitable and valid general-purpose method at present available for the determination of plasma bilirubin.

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Hypercholesterolaemia and diabetes mellitus

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SYNOPSIS An association of essential hypercholesterolaemia and diabetes mellitus is postulated. Case records are included to illustrate hypercholesterolaemia secondary to acute diabetes mellitus, and essential hypercholesterolaemia, independent of, but accompanying diabetes mellitus. Whereas in the former the serum cholesterol returns to normal and its attendant clinical manifestations resolve when the diabetes is controlled, this is not so in the latter type.

Evidence is presented to show that impaired carbohydrate tolerance is not a feature of essential hypercholesterolaemia.

Abnormalities of lipid metabolism associated with uncontrolled diabetes mellitus have been assumed to be secondary to the disordered carbohydrate metabolism, an assumption apparently confirmed by the fall in serum cholesterol which usually accompanies control of the diabetes mellitus (Thannhauser, 1950; Haymond and Berry, 1954). Observations on diabetic children in fair or good diabetic control showed that mean serum levels of β lipoproteins and cholesterol were significantly higher in the children with blood sugar levels above 200 mg. per 100 ml. than in those with blood sugars below this figure (Wolff and Salt, 1958).

Failure of an elevated serum cholesterol level to return to normal when diabetic control has been established has been stated to be of serious prognostic importance (Cantarow and Trumper, 1955). The possibility that this continued hypercholesterolaemia may represent an independent metabolic abnormality appears to have received relatively little attention.

The case histories which follow serve to illustrate these two patterns of hypercholesterolaemia which may be encounted in patients with diabetes mellitus.

CASE REPORTS

CASE 1 A woman aged 29 years presented with one year's history of polydipsia, polyuria, lassitude, and weight loss; an augmented appetite may have resulted in a high fat intake.

Examination revealed a cutaneous xanthoma diabeticorum and very striking lipaemia retinalis. Diabetes mellitus was confirmed by the finding of heavy glycosuria with acetonuria, a fasting blood sugar of 205 mg. per 100 ml. rising to 368 mg. per 100 ml. two hours after 100 g. glucose by mouth. The fasting serum was milk white in appearance and the serum cholesterol 1,458 mg. per 100 ml. Treatment with insulin resulted in a rapid clearance of the xanthomata, lipaemia retinalis, and the plasma lipaemia, the serum cholesterol falling eventually to 166 mg. per 100 ml. (Table I). This patient has been described in greater detail elsewhere (Bloom and Crowley, 1956).

TABLE I

SERUM CHOLESTEROL LEVELS IN CASES 1 AND 2 AFTER STABILIZATION WITH INSULIN

	Serum Cholesterol (mg. per 100 ml.)	
	Case 1	*Case 2*
Initially	1,458	1,011
After stabilization with insulin		
Days 7	550	—
Days 10	500	—
Days 14	330	420
Days 17	350	—
Days 21	250	—
Days 24	270	225
Days 28	300	203
Weeks 7	166	—
Weeks 24	—	—

CASE 2 A youth of 17 years of age was admitted with one month's history of polydipsia, polyuria, weight loss, and weakness.

Examination showed a young man of poor nutrition with clinical dehydration. Ophthalmoscopy revealed striking lipaemia retinalis; there were no skin xanthomata.

Urine analysis showed severe glycosuria and ketonuria; the blood sugar was 350 mg. per 100 ml. The plasma was milk white and the total serum cholesterol 1,011 mg. per 100 ml. Response to insulin was prompt, the diabetic state coming under control within 48 hours; the lipaemia retinalis became rapidly less marked and the retinal vessels were normal again in about 14 days, coincident with the fall in plasma lipaemia and hypercholesterolaemia (Tables I and IA).

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TABLE IA

RESPONSE OF PLASMA LIPIDS TO INSULIN THERAPY IN CASE 2

Day of Disease	Neutral Fat (mg./100 ml.)	Cholesterol (mg./100 ml.)	Phospholipids (mg./100 ml.)	Total Lipids (mg./100 ml.)
1	7,858 (0-200)	1,011 (150-300)	1,217 (60-350)	10,095 (570-820)
14	318 (0-200)	420 (150-300)	411 (60-350)	1,149 (570-820)
28	323 (0-200)	225 (150-300)	273 (60-350)	821 (570-820)

In both patients the extreme hyperlipaemia with hypercholesterolaemia which very occasionally accompanies acute diabetes mellitus was well illustrated. Eruptive xanthomata in one and striking lipaemia retinalis in both were the main clinical signs of this metabolic abnormality. Extreme lipaemia was confidently predicted in Case 2 following routine fundus examination on admission.

The clinical signs and hypercholesterolaemia resolved promptly in both patients with control of the diabetes mellitus.

CASE 3 A woman of 65 years, complaining of pruritus vulvae, polydipsia, and polyuria, was referred to hospital for investigation. The patient had well-developed xanthoma palpebrum; there was no evidence of myxoedema, hepatic, or renal disease.

Urinary examination showed heavy glycosuria but no proteinuria. Blood sugar was 198 mg. per 100 ml. fasting and 259 mg. per 100 ml. two hours after ingestion of 50 mg. of glucose. The serum was clear and the serum cholesterol 400 mg. per 100 ml. A low calorie diet failed to control glycosuria, but the patient refused insulin.

Four years later the patient returned complaining of retrosternal pain and at this time an electrocardiogram showed a posterior myocardial infarction. She was then treated with carbutamide which controlled the diabetes initially, but this was not accompanied by a fall in serum cholesterol.

A low fat diet, sitosterol, and finally a 'corn oil' diet were employed in an attempt to reduce the raised serum cholesterol (Fig. 1).

CASE 4 A woman of 59 years was referred to hospital because of retrosternal pain on effort. Examination showed a marked corneal arcus but no other significant abnormality. The electrocardiographic tracing was consistent with a posterior myocardial infarction.

Six months later the patient returned complaining of thirst, polyuria, and pruritus vulvae. Urine analysis showed glycosuria, and the fasting blood sugar was 180 mg. per 100 ml. A glucose tolerance test performed more recently showed a blood sugar of 223 mg. per 100 ml. two hours after ingestion of 5 g. glucose. The serum cholesterol was 530 mg. per 100 ml.; the fasting serum was clear.

Glycosuria was rapidly controlled by a low calorie

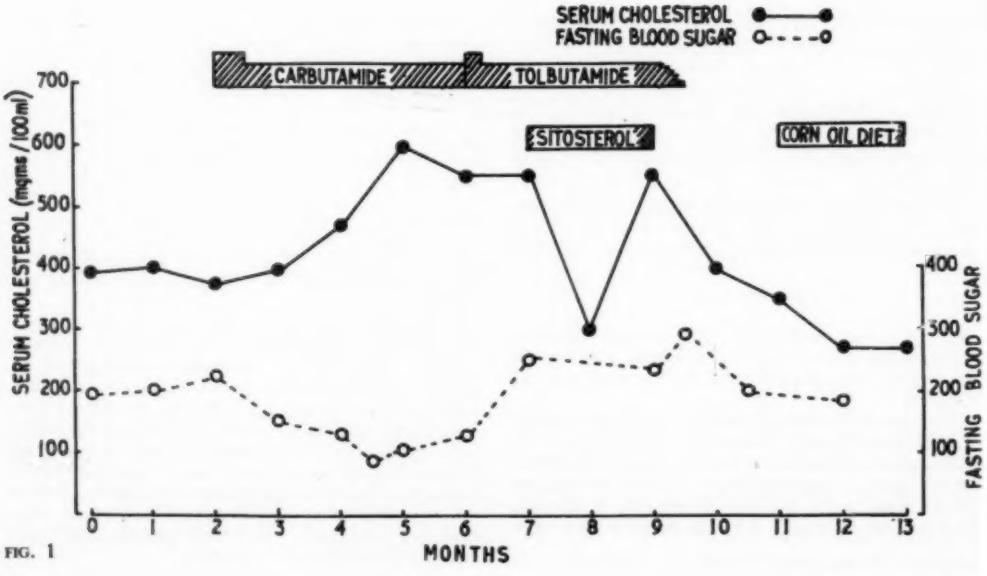

FIG. 1

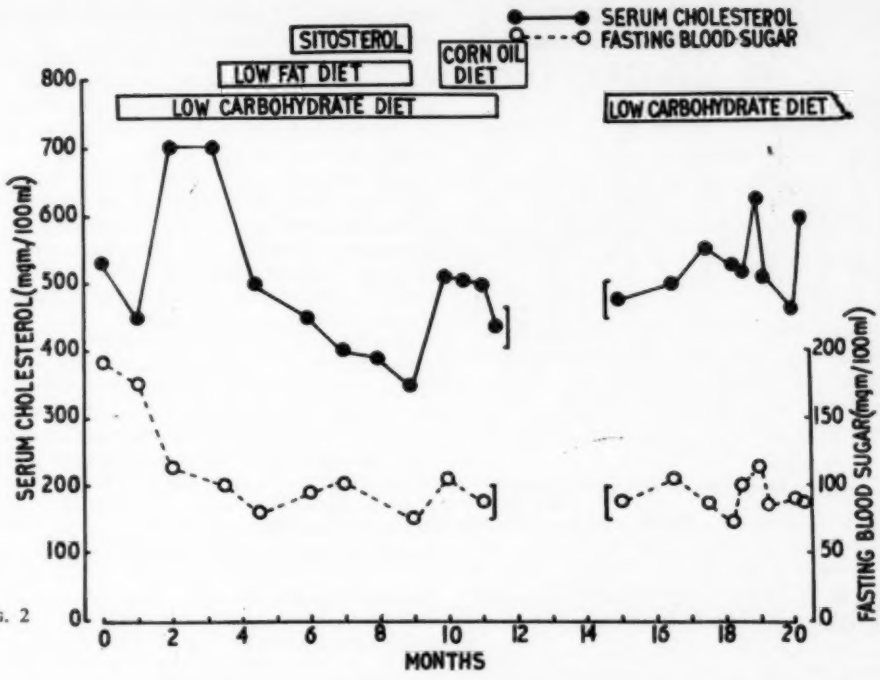

FIG. 2

diet, but control of the diabetes was not accompanied by a fall in the serum cholesterol. A low fat diet, sitosterol, and subsequently a 'corn oil' diet were used in an unsuccessful attempt to reduce the raised serum cholesterol level (Fig. 2).

Both patients demonstrate the association of essential hypercholesterolaemia with diabetes mellitus; in each case levels of serum cholesterol were recorded which were well above those quoted as normal for the sex and age group concerned by Keys, Mickelsen, Miller, Hayes, and Todd (1950). The essential nature of this hypercholesterolaemia was supported by the presence of xanthelasma palpebrum in Case 3, which remained uninfluenced by control of the diabetes, and the presence of a marked corneal arcus in Case 4, which is not a recorded feature of secondary hypercholesterolaemia.

That these cases represented examples of essential hypercholesterolaemia and not hyperlipaemia was supported by the fact that in both the values for neutral fats and phospholipids were normal, and that in neither case was the fasting serum lactescent.

There is little evidence that impairment of carbohydrate metabolism occurs with undue frequency in essential hypercholesterolaemia. During an investigation of families with essential hypercholesterolaemia (Harris-Jones, Jones, and Wells, 1957), fasting blood sugar estimations were obtained in patients with hypercholesterolaemia and their near relatives. Whereas such fasting blood sugar levels alone cannot exclude impaired carbohydrate metabolism, they remained normal throughout a wide range of serum cholesterol levels (Fig. 3). Glucose tolerance tests, using the standard 50 g. oral method, were performed on six patients with hypercholesterolaemic xanthomatosis, and were found to be

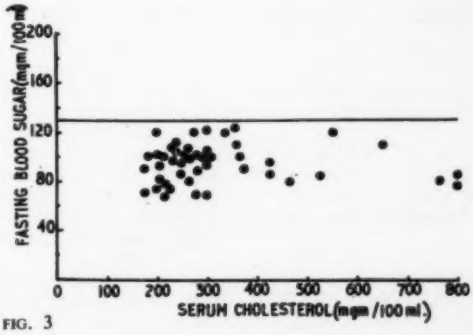

FIG. 3

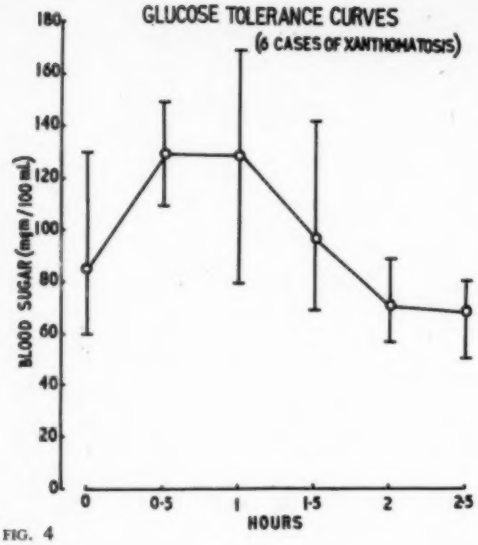

FIG. 4

normal (Fig. 4). This is in agreement with a similar investigation by Wilkinson, Hand, and Fliegelman (1948).

DISCUSSION

Hypercholesterolaemia accompanying uncontrolled diabetes mellitus is well recognized.

Increased fatty acid metabolism consequent on insulin deficiency results in an increase in concentration of the intermediary products of fat metabolism. Therefore, an increased supply of 'acetate' becomes available for synthesis into keto-acids and cholesterol (Mookerjea and Sadhu, 1957). As would be expected, the abnormal cholesterol metabolism is modified when carbohydrate metabolism is restored towards normal by treatment. This is expressed by the resolution of skin xanthomata, and rapid fall of the serum cholesterol, which follow stabilization of the diabetes.

Less is known concerning the metabolic defect responsible for essential hypercholesterolaemia. It is an inborn error of metabolism transmitted as an incomplete dominant trait, and detected most commonly in the middle age groups.

Inheritance of two independent metabolic defects may be encountered, as illustrated by the association of diabetes mellitus and gout (Weiss, Segaloff, and Moore, 1957), or hypercholesterolaemia and hyperuricaemia (Harris-Jones, 1957). An association of diabetes mellitus and hypercholesterolaemia would not therefore appear improbable.

In this event one would not expect the clinical manifestation of hypercholesterolaemia, or the serum cholesterol to be necessarily affected by control of the diabetic state. As will be observed in Cases 3 and 4, in which the hypercholesterolaemia was considered to be an essential defect, reduction of the fasting blood sugar was in fact associated with a rise in the serum cholesterol. No valid hypothesis can be offered for this apparent paradoxical behaviour of the serum cholesterol.

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Multiple plasma enzyme activities in liver disease

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SYNOPSIS The measurement of the plasma activities of glutamic-oxaloacetic and glutamic-pyruvic transaminases, aldolase, cholinesterase, and isocitric, lactic, and phosphogluconic dehydrogenases in random samples of blood was found to be of no value in the differential diagnosis of hepatitis, obstructive jaundice, hepatic cirrhosis, and neoplastic conditions involving the liver. Serial determinations of the enzyme activities provided useful information about the course of certain hepatic disorders, particularly acute viral hepatitis.

In recent years an increasing amount of attention has been given to the fluctuations of blood enzyme activities in liver disease. Numerous workers (cf. Greenberg and Harper, 1960) have shown that one or more of the many enzymes present in serum may be abnormal in various types of hepatic dysfunction. It seemed possible that characteristic patterns of blood enzyme abnormalities might be distinguished for particular disorders of the liver. We have therefore studied the variation in the plasma activities of several enzymes belonging to four different groups (glycolytic, dehydrogenases, transaminases, and esterases) in patients with hepatitis, obstructive jaundice, cirrhosis, and neoplastic conditions involving the liver. The determinations were performed on single random specimens of blood and also on serial samples obtained from selected patients.

MATERIALS AND METHODS

PLASMA ENZYME ACTIVITIES The enzyme activities measured, together with the analytical methods and incubation temperatures used, are given in Table I.

Two difficulties arise with respect to the units employed by various workers to express an enzyme activity. The principal source of possible error is that the activity of an enzyme may be determined by different methods involving the definition of different units and these may not be interconvertible. Thus, two or even more, normal ranges and abnormal responses for a particular enzyme may be encountered in the literature. The units employed in the present work are those defined in the analytical methods listed in Table I and may not necessarily be equivalent to units defined by other workers. A second difficulty relates to the volume of plasma or serum used to define the normal range. Enzyme activities are usually expressed in terms of 1 ml. of plasma or serum. However, alkaline phosphatase and cholinesterase activities have been defined for many years in terms of 100 ml. of plasma and serum, and it would cause some confusion to alter this long-established practice. Enzyme activities are extremely dependent upon temperature but there is, as yet, no general agreement about either an internationally standard temperature at which enzyme activities should be measured or an appropriate correction factor by which results obtained at different temperatures could be interconverted. We have therefore employed two widely used incubation temperatures (25° and 37°C.) and have not attempted to apply corrections.

The methods for the determination of aldolase, the two transaminases, alkaline phosphatase, and cholinesterase were colorimetric procedures and the final determination of the optical densities were made with a Hilger Spekker absorptiometer. The dehydrogenase estimations were based on the conversion of the reduced forms of either diphosphorypyridine or triphosphopyridine nucleotides to their corresponding oxidized forms. These latter reactions were measured by the changes in optical density at 340 mμ using a Hilger Uvispek spectrophotometer. The normal range for each plasma enzyme activity was determined by analysing random blood specimens, collected into heparinized bottles, from 50 normal adult subjects.

Other biochemical examinations included the estimation of serum bilirubin (Malloy and Evelyn, 1937), the serum thymol turbidity test (Maclagen, 1944), and qualitative tests for urinary bilirubin, urobilinogen, and urobilin (Harrison, 1957).

PATIENTS The plasma enzyme activities and other liver function tests were performed on blood samples taken at random from 53 patients. They included patients in the

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TABLE I

PLASMA ENZYME ACTIVITIES MEASURED

Group Activity	*Enzyme*	*Incubation Temperature (°C.)*	*Analytical Method*
Glycolytic	Aldolase	37	Sibley and Lehninger (1949)
Transaminase	Glutamic-oxaloacetic	37	Reitman and Frankel (1957)
	Glutamic-pyruvic		
Dehydrogenase	Lactic	25	Kaltenbach *et al.* (1957)
	Isocitric		Wolfson and Williams-Ashman (1957)
	Phosphogluconic		
Esterase	Alkaline phosphatase	37	Powell and Smith (1954)
	Cholinesterase		De la Huerga, Yesinick, and Popper (1952)

wards and from the out-patient departments referred to the laboratory for the investigation of liver function. The patients were divided into the groups shown in Table II and the clinical diagnosis was subsequently confirmed by needle biopsy of the liver, by examination of bone marrow or lymph nodes in the case of five patients,

TABLE II

PATIENTS INVESTIGATED

Clinical Group		*Number of Patients*	
Infective hepatitis			7
Obstructive jaundice	Gall stones	12	25
	Carcinoma	7	
	Biliary cirrhosis	6	
Hepatic cirrhosis			12
Neoplastic conditions	Secondary carcinoma	6	9
	Reticulosarcoma	1	
	Hodgkin's disease	2	

at operation for 24 patients, and at necropsy for 12 who died. For the remainder the diagnosis was considered to be established by a combination of the clinical evidence and the results of conventional liver function tests for urinary bile pigments, serum bilirubin, and serum thymol turbidity. Serial specimens were obtained at weekly intervals from a number of patients selected from those contained in Table II. Analyses for the plasma enzyme activities and other liver functions were continued as far as possible throughout the course of the disease. The cases studied included a mild infective hepatitis of short duration, a more severe infective hepatitis of prolonged duration, a cirrhosis with portal hypertension, a carcinoma of the bronchus with massive secondary deposits in the liver, and a case of Hodgkin's disease involving the liver before and during treatment with nitrogen mustard and *x*-ray therapy.

RESULTS

NORMAL SUBJECTS The mean values and normal ranges for the plasma enzyme activities under our laboratory conditions were determined in 50 normal subjects and the results are given in Table III.

The results for the enzyme activities are in good agreement with those observed in normal subjects by the great majority of other workers (King, 1959),

TABLE III

NORMAL VALUES FOR PLASMA ENZYME ACTIVITIES

Enzyme	*Mean*	*Range*	
Aldolase	4	1- 8	per ml. of plasma
Glutamic-oxaloacetic transaminase	14	6- 31	
Glutamic-pyruvic transaminase	9	0- 21	
Lactic dehydrogenase	19	6- 42	
Isocitric dehydrogenase	112	40-240	
Phosphogluconic dehydrogenase	134	40-280	
Alkaline phosphatase	8	4- 13	per 100 ml. of plasma
Cholinesterase	234	121-317	

except that the mean value for lactic dehydrogenase obtained in the present work is lower than the mean of 30·6 units reported by Kaltenbach, Becker, and Bernstein (1957) who used the same method of estimation.

RANDOM SPECIMENS FROM PATIENTS WITH LIVER DISEASE The results are given in Table IV, expressed as the percentages of abnormal results for each plasma enzyme activity found in each clinical group and also for the total number of patients.

All the clinical groups showed appreciable percentages of abnormal values for the enzyme activities and no characteristic patterns of plasma enzyme abnormalities could be distinguished for the types of liver disease studied in the present work. The results of the thymol turbidity test offered more positive guidance in the differential diagnosis of infective hepatitis and obstructive jaundice than any of the plasma enzyme activities studied.

SERIAL ESTIMATIONS OF PLASMA ENZYME ACTIVITY These were done in several conditions affecting the liver.

Infective hepatitis Two cases of infective hepatitis, both in young male adults, were studied. The first example was that of a mild hepatitis of relatively short duration. The patient gave a history of one week of nausea and some vomiting, but was not seen until he had become clinically jaundiced. The serum bilirubin level never exceeded 5 mg. per 100 ml. and returned to normal within five weeks. He was

TABLE IV

PLASMA ENZYME ACTIVITIES IN RANDOM BLOOD SAMPLES[1]

Clinical Group	*Plasma Enzyme Activities*								
	Aldolase	*Glutamic-oxaloacetic Transaminase*	*Glutamic-pyruvic Transaminase*	*Lactic Dehydrogenase*	*Isocitric Dehydrogenase*	*Phosphogluconic Dehydrogenase*	*Alkaline Phosphatase*	*Cholinesterase*	*Serum Thymol Turbidity Test*
Hepatitis	85	85	85	55	85	30	55	55	55
Obstructive	35	80	60	20	30	20	95	35	0
Cirrhosis	65	65	50	40	50	50	75	25	50
Neoplastic	55	65	55	65	45	20	45	35	10
All cases	55	75	60	40	45	25	75	40	25

[1]Results are expressed as percentages of abnormal values for each enzyme activity.

TABLE V

PLASMA ENZYME RESULTS IN MILD HEPATITIS OF SHORT DURATION

Day	*Bilirubin (mg./100ml.)*	*Aldolase*	*Glutamic-oxaloacetic Transaminase*	*Glutamic-pyruvic Transaminase*	*Lactic Dehydrogenase*	*Isocitric Dehydrogenase*	*Phosphogluconic Dehydrogenase*	*Alkaline Phosphatase*	*Cholinesterase*
1	4·7	42	830	730	36	1,040	680	16	98
8	4·8	8	220	680	25	140	80	26	47
15	1·7	4	50	130	13	170	60	18	250
22	1·5	6	50	60	18	220	120	14	220
29	1·2	3	34	40	25	150	100	10	310
36	0·9	6	25	18	16	100	80	6	250
43	0·5	4	20	10	20	120	160	6	280

not acutely ill and made an uneventful recovery. Serial estimations of the plasma enzyme activities and other liver function tests were made at weekly intervals for six weeks and the results are shown in Table V.

The results show that all the plasma enzyme activities, with the exception of the lactic dehydrogenase, showed abnormal values when the patient was first seen. The aldolase, glutamic-oxaloacetic transaminase, isocitric dehydrogenase, phosphogluconic dehydrogenase, and cholinesterase activities returned to normal before the serum bilirubin decreased from its maximum concentration, whereas the alkaline phosphatase and glutamic-pyruvic transaminase activities decreased more slowly. The liver damage therefore appeared to be reflected in the high proportion of abnormal enzyme activities. However, this patient was not seen in the pre-icteric stage of the disease, when damage to the liver parenchyma had probably occurred and the time relation of the plasma enzyme activity disturbances to a rising serum bilirubin concentration could not be established.

The second case of infective heptatitis was studied in the pre-icteric stage, *i.e*, before clinical jaundice had supervened. Abnormally high values for the activities of the two transaminases were found during the period that the total serum bilirubin concentration increased from normal to 7 mg. per 100 ml., at which point all the other enzyme activities, with the exception of lactic dehydrogenase, became abnormal. Thus, the plasma transaminase activities gave an indication of liver damage before overt jaundice occurred. The detailed results for this patient are shown in Fig. 1.

As in the case of the mild hepatitis, all the enzyme activities, except lactic dehydrogenase, showed abnormal values during the period that the jaundice was increasing. The serum bilirubin level reached 26 mg. per 100 ml., remained above 20 mg. for several weeks, and then slowly returned to normal. The majority of the abnormal enzyme activities returned to normal before the bilirubin had reached its peak but the alkaline phosphatase tended to remain slightly high. On the twenty-sixth day of the illness the patient had a further bout of nausea and vomiting and was considered on clinical grounds to have suffered a relapse. This episode was not accompanied by any further changes in the bilirubin concentration, in the thymol turbidity, alkaline phosphatase, or in the tests for urinary pigments. However, the glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, isocitric dehydrogenase, and phosphogluconic dehydrogenase showed increased activities and the cholinesterase showed abnormally low values coinciding with the clinical relapse. The aldolase, which showed abnormal values at the beginning of the disease, failed to show this

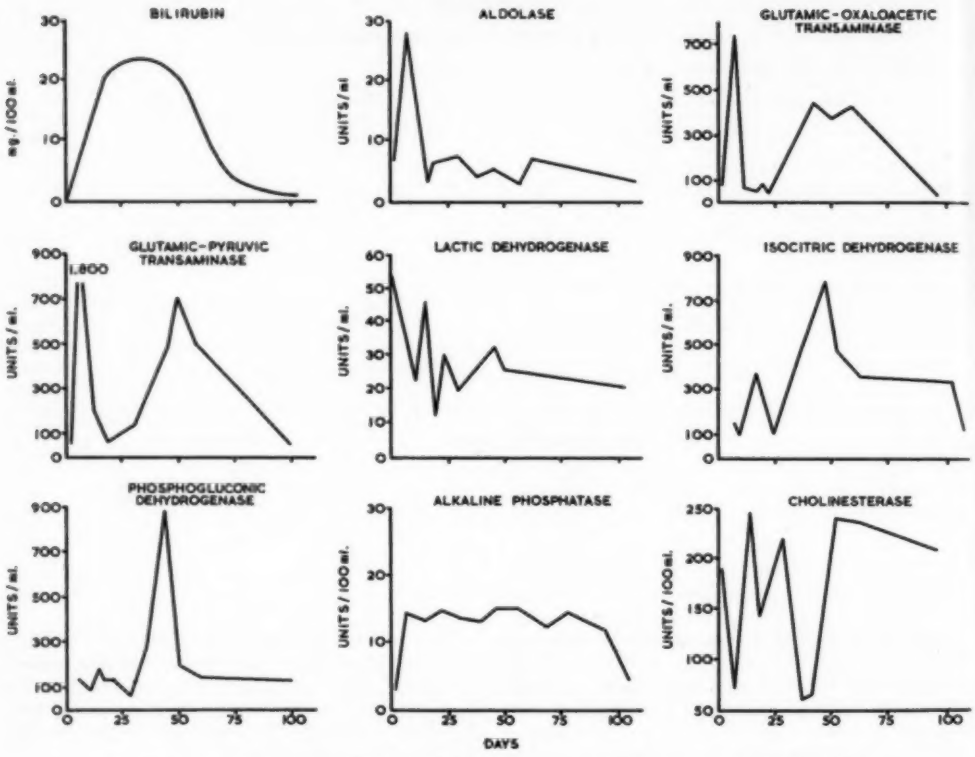

FIG. 1. *Plasma enzyme activities and bilirubin concentrations in severe infective hepatitis.*

secondary response. The abnormal plasma enzyme activities all returned to normal with the eventual subsidence of the jaundice. It therefore appears that some of the plasma enzymes (glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, phosphogluconic dehydrogenase, isocitric dehydrogenase, and cholinesterase) were more sensitive indices of further liver damage during a clinical relapse than were the more conventional liver function tests such as the alkaline phosphatase, thymol turbidity, and urine pigments. The serial results with lactic dehydrogenase were very inconsistent in both cases of hepatitis and aldolase rarely showed abnormal values.

Cirrhosis A woman, aged 55, with cirrhosis of the liver and portal hypertension, had numerous episodes of severe haemorrhage from oesophogeal varices which necessitated repeated blood trans-

TABLE VI

PLASMA ENZYME ACTIVITIES IN CIRRHOSIS

Day	*Bilirubin (mg./100ml.)*	*Aldolase*	*Glutamic-oxaloacetic Transaminase*	*Glutamic-pyruvic Transaminase*	*Lactic Dehydrogenase*	*Isocitric Dehydrogenase*	*Phosphogluconic Dehydrogenase*	*Alkaline Phosphatase*	*Cholinesterase*
1	4·8	1	102	60	132	80	150	86	66
16	5·2	6	130	56	120	60	200	70	80
37	5·4	8	90	26	70	80	80	66	190
65	6·6	4	125	80	25	120	130	73	65
105	4·2	16	80	74	28	520	500	80	170
120	5·5	9	120	155	20	170	220	32	90

fusions. Serial determinations of the plasma enzyme activities were performed during a period of four months from her first attendance as an out-patient until her death as a result of haemorrhage. The results (Table VI) show that the two transaminases and the alkaline phosphatase remained consistently high during the course of the disease, as did the serum bilirubin. The cholinesterase exhibited a fluctuating behaviour and the dehydrogenases and aldolase showed isolated abnormal values.

Hepatic metastases A man, aged 69, died of carcinoma of the bronchus with massive secondary deposits in the liver, enzyme studies being made during a period of 23 days before his death (Table VII). The serum bilirubin rose to a fairly steady level of 6 mg./100 ml., the transaminases, isocitric dehydrogenase, and lactic dehydrogenase remained consistently high and the cholinesterase was abnormally low on two occasions. The alkaline phosphatase and aldolase levels were raised until the terminal stages of the illness when they fell to normal levels. This terminal fall in the alkaline phosphatase was also observed in several other cases of hepatic metastases and also of cirrhosis.

Hodgkin's disease involving the liver A man, aged 42, with Hodgkin's disease and severe ascites, was treated with nitrogen mustards and deep *x*-ray therapy and showed clinical improvement. The plasma enzyme activities were measured on several occasions before, during, and after the treatment (Table VIII).

The abnormal values found for glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, and cholinesterase returned to normal with clinical improvement whereas the alkaline phosphatase remained high although somewhat decreased after treatment. The dehydrogenase values were never found to be high.

TABLE VII

PLASMA ENZYME ACTIVITIES IN HEPATIC METASTASES

Day	*Bilirubin (mg./100ml.)*	*Aldolase*	*Glutamic-oxaloacetic Transaminase*	*Glutamic-pyruvic Transaminase*	*Lactic Dehydrogenase*	*Isocitric Dehyrogenase*	*Phospho-gluconic Dehydrogenase*	*Alkaline phosphatase*	*Cholin-esterase*
1	0·6	20	25	12	67	240	—	5	146
6	0·7	18	36	30	45	840	170	36	130
11	4·5	62	220	113	100	300	280	43	8
17	6·0	18	270	50	105	360	140	65	45
18	5·8	5	140	30	170	400	72	54	120
22	6·6	8	180	72	70	720	160	6	160

DISCUSSION

The present work was designed to answer two questions. First, does the analysis of random blood specimens for multiple enzyme activities provide a useful laboratory aid in the differential diagnosis of liver disease? This question was considered to be of some practical importance since clinical laboratories are frequently presented with such specimens. In Table IV the results have been expressed as the percentages of abnormal values found for each enzyme activity in each clinical group. All the clinical groups showed appreciable numbers with abnormal values for each enzyme and it does not appear that the incorporation of these particular enzyme measurements in the normal battery of liver function tests is justified. Sterkel, Spencer, Wolfson, and Williams-Ashman (1958) have stated that serum isocitric dehydrogenase is seldom abnormal in extrahepatic obstruction but its activity was elevated in 30% of cases of obstructive jaundice in the present series and compared unfavourably with the thymol turbidity test in the differentiation of acute hepatitis and posthepatic jaundice. The well-known combination of an alkaline phosphatase and thymol turbidity

TABLE VIII

PLASMA ENZYME RESULTS IN HODGKIN'S DISEASE

Day	*Treatment*	*Bilirubin (mg./100ml.)*	*Glutamic-oxaloacetic Transaminase*	*Glutamic-pyruvic Transaminase*	*Alkaline Phosphatase*	*Cholinesterase*
1		3·0	80	26	105	45
3		5·7	90	105	132	50
5	Nitrogen mustards and *x*-ray therapy	13·2	145	130	148	64
6		1·9	30	10	64	183
7		1·2	25	7	72	100

gives as much, if not more, guidance in the differential diagnosis of liver disease than would any more elaborate combination which included one or more of the plasma enzyme activities measured in the present work.

The second question was concerned with the possible value of serial measurements of multiple plasma enzyme activities in assessing the course of particular hepatic disorders. The results suggest that such estimations may give useful information but that it is not necessary to measure all the enzyme activities. Thus, the fluctuations in either of the transaminases (glutamic-oxaloacetic or glutamic-pyruvic) reflected the major changes in any of the other enzymes studied. Abnormal values for the transaminases were obtained in acute hepatitis before overt jaundice had supervened and they appeared to be sensitive indices of a further episode of parenchymal damage during a severe hepatitis (Fig. 1). The abnormal values quickly returned to normal in an uncomplicated case of hepatitis (Table III) and their persistently raised levels in the cases of cirrhosis (Table VI) and of hepatic metastases (Table VII) indicated continuing liver damage. A further point of interest was the return to normal of the high transaminase values in the patients with Hodgkin's disease who responded favourably to treatment (Table VII). The serial estimation of serum transaminases would therefore be a valuable adjunct to the other conventional liver function tests in the assessment of the course of some types of liver disease.

We are indebted to our clinical colleagues for allowing us to investigate the patients under their care.

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Soluble cerebral proteins in normal and oedematous brain

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SYNOPSIS The soluble cerebral proteins of cortex and white matter from normal brains of adults and infants and of oedematous brain tissue have been subjected to starch electrophoresis. The protein patterns with their nitrogen, lactic dehydrogenase, and glutamic oxalacetic transaminase contents were estimated. The normal patterns of these substances have been compared with the findings of workers using other methods of electrophoresis.

Oedematous brains have been shown to possess an albumin band with an increase in lactic dehydrogenase in some globulin bands. These findings are discussed in relation to the mode and site of production of oedema.

The electrophoretic pattern of the proteins of the various body fluids has been and still is being extensively studied. Relatively few similar examinations of the soluble cerebral proteins have been performed in normal brain and even fewer in disease processes.

Robertson (1960) has recently recorded his findings from normal brains and made some comparison with the results published by a few other authors. Originally paper electrophoresis was carried out by Kaps (1954), Palladin (1957), and Tomonaga (1959) and this was the method used by Robertson. Van Sande, Karcher, and Lowenthal (1959) have used agar as well as paper electrophoresis, while Kessel (1959) has examined tissue proteins from the liver and kidney by agar electrophoresis and Schwarzmann (1959) has applied starch electrophoresis to extracts of the liver and stomach.

This paper records the results of the electrophoresis of soluble cerebral protein from normal brains and from oedematous cerebral tissue utilizing the starch technique of Smithies. Further, the amounts of lactic dehydrogenase (L.D.H.), glutamic oxalacetic transaminase (G.O.T.), and of nitrogen in the various protein fractions have been estimated. A somewhat similar but less detailed study of the brains of young infants has been performed. A few of the earliest studies have already been briefly mentioned (Cumings, 1959).

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MATERIAL AND METHODS

Brains have been obtained at necropsy performed less than 24 hours after death and portions of normal and abnormal cortex and cerebral white matter carefully dissected out immediately. Fourteen portions of frontal lobe, five of temporal, nine of parietal, 17 of occipital, and two each from the basal ganglia and the pons were initially examined. The areas showing oedema in brains from patients with cerebral tumours were the frontal and temporal lobes (two each) and the occipital and parietal lobes (one each).

For the enzyme studies further brains were used, in three of which only L.D.H. was estimated and 10 brains in which L.D.H., G.O.T., and nitrogen were estimated. One oedematous brain was examined for L.D.H. only, while five such brains were tested for their contents of L.D.H., G.O.T., and nitrogen, and in one infarcted brain from vascular occlusion all these examinations were made. The brains of children at full term, at 2, 3, and 8 months and at 6 years were examined by electrophoresis as well as for enzymes.

After separation of white matter and of cortex 5 g. of the former was extracted with 10 ml. of 0·3 M. sucrose. Either similar amounts or more frequently 5 g. of cortex was extracted with 5 ml. of sucrose of the same molar strength. The extracts after centrifugation at 12,000 *g.* in a refrigerated centrifuge at 0° to 4°C. were concentrated by negative pressure at 4°C., recentrifuged if necessary, and, after measurement of the volume, a portion was examined by starch electrophoresis.

The method employed was that of Smithies (1955), using both buffer and staining procedures as recommended by him. Electrophoresis was carried out overnight (16-18 hours) at a voltage of 120 and with a current

of 0·44 MA. per cm. width. The gel was marked along one longitudinal edge with a serrated metal cutter so that after the staining of one longitudinal section for protein, the fractions could be accurately located in the remaining unstained gel. This half of the gel was then subdivided into from six to 10 portions in which were specific protein bands. Each portion was placed within a small glass cylinder upon a fritted filter sealed within the tube and the whole placed within a conical centrifuge tube. After freezing solid overnight, the tubes were centrifuged at about 2,000 r.p.m. The volume of the protein-containing fluid was measured and aliquots were examined for L.D.H., G.O.T., and for total nitrogen, which substances were also estimated in the original concentrate.

Lactic dehydrogenase was estimated by the method of Wróblewski and LaDue (1955), G.O.T. by King (1958), and total nitrogen by Greenberg's method (1929), and a microKjeldahl method was used to confirm the degree of accuracy, which was within 2%.

RESULTS

The number and site of the various protein fractions has varied from case to case and area to area. Usually between two and four bands migrate cathodally as the γ globulins of blood sera, while from seven to 10 bands migrate towards the anode. Almost invariably two and even three pre-albumin bands are present. Figure 1 is a photograph of 10 starch gels from various areas of both white and grey matter. Definite differences can be noted between the different areas of the cerebrum; for instance, more bands are present in the occipital lobes. However, there is often slightly more albumin and pre-albumin in the cortex than in the white matter, as well as more protein in fractions 3 and 4 migrating anodally. However, the actual amounts of protein can more readily be seen in Figs. 5 and 6 which are discussed later.

The findings in the oedematous brains are illustrated in Fig. 2, together with those in sera from three of the patients. Two points are striking; first, there is a very marked content of albumin in the cerebral white matter exactly matching the site of serum albumin. This band does not obscure the pre-albumin band. Secondly, the cerebral cortex does not always show this albumin; in fact, in only half of the brains examined could this feature be demonstrated. These changes can also be seen in Figs. 7 and 8 which are described later.

Figs. 3 and 4 show the results obtained on electrophoresis of the soluble cerebral proteins from young infants. The protein pattern is not so well demonstrated in that the bands tend not to be so well separated, and fewer distinct bands can be found. However, more protein is extractable from these brains than from those of adults as will be seen from Tables I and II.

Considering the enzyme studies, examples of the results are seen in Figs. 5 and 6. These illustrate the percentage protein contents of the bands and the relative distribution of L.D.H. and G.O.T. in the bands. The amounts of these substances in the original concentrate related to 1 g. of fresh brain are also given. The quantity of nitrogen per g. of fresh brain was fairly constant for both white matter and cortex, usually being between 0·15 and 0·25 mg. for

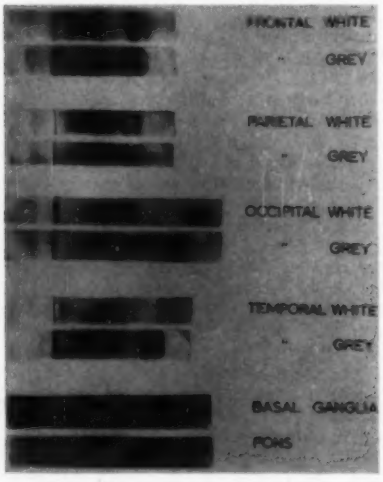

FIG. 1

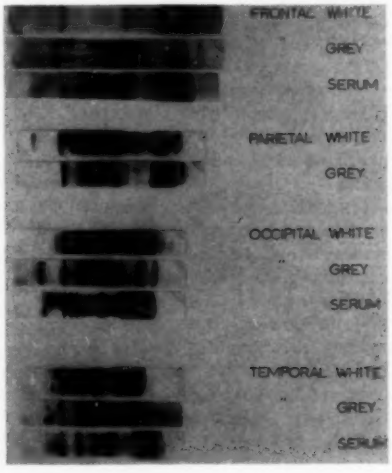

FIG. 2

FIG. 1. *Soluble cerebral proteins in normal brain.*

FIG. 2. *Soluble cerebral proteins in oedematous brain.*

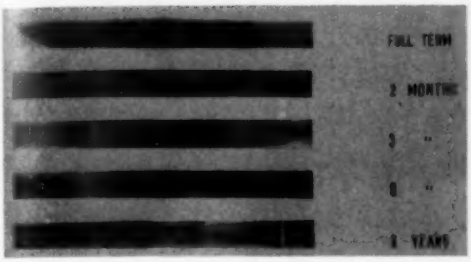

FIG. 3. *Soluble cerebral proteins in cerebral white matter.*

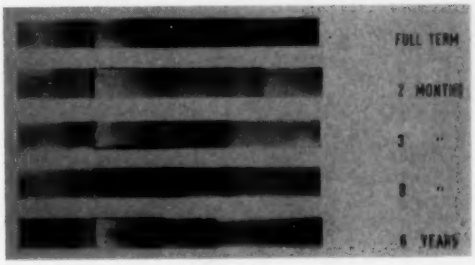

FIG. 4. *Soluble cerebral proteins in cortex.*

the former and 0·5 and 0·75 for the latter. Considerable amounts of L.D.H. were located within the first four protein bands travelling anodally from the site of insertion, while none was ever present in the γ globulin, albumin, or pre-albumin fractions. By contrast, G.O.T. was present in small amounts and was distributed somewhat unevenly over all the protein bands.

Similar studies of oedematous brain are illustrated in Figs. 7 and 8. There is seen to be an increased total protein as indicated by nitrogen content and by the presence of a marked albumin band in both white matter and cortex. There is a definite increase in L.D.H. which is present in the same bands as in a normal brain, while no increase in G.O.T. is seen. Other samples of oedematous brain examined showed very similar results, although, on two occasions only, very small quantities of L.D.H. were found in the albumin band. In no case was the total amount of G.O.T. increased. The infarcted brain showing slightly increased water content with a small albumin band in both cortex and white matter yet demonstrated no alteration from the normal in content or in distribution of these two enzymes (Figs. 9 and 10).

The brains of infants showed results that are given in Tables I and II. In general there is a steady increase in both L.D.H. and G.O.T. per mg. of nitrogen up to 8 months of age when figures similar to those of the adult were obtained. The lower L.D.H. at 6 years in both cortex and white matter is unexplained and may be a fortuitous result, or it may be that the figure at 8 months is too high, for this infant died of an acute pyogenic infection.

DISCUSSION

Examinations of the soluble cerebral proteins were carried out first by paper electrophoresis by Kaps (1954). Hofmann and Schinko (1956), Robertson (1957), and Karcher, Lowenthal, and van Sande (1958), and some six or so different bands which

TABLE I

ENZYMES IN FRESH CEREBRAL WHITE MATTER

Age	*% Water Content*	*mg. N_2 per g. Brain*	*G.O.T. in Units*		*L.D.H. in Units*	
			per g. Brain	*per mg. N_2*	*per g. Brain*	*per mg. N_2*
Stillborn	87·4	0·82	29	35	6,160	7,510
2 months	86·0	0·20	7	35	3,130	15,600
3 months	84·1	0·31	19	61	4,000	12,900
8 months	78·3	0·74	52	70	29,000	39,000
6 years	71·0	0·82	86	105	3,600	4,400

TABLE II

ENZYMES IN FRESH CEREBRAL CORTEX

Age	*% Water Content*	*mg. N_2 per g. Brain*	*G.O.T. in Units*		*L.D.H. in Units*	
			per g. Brain	*per mg. N_2*	*per g. Brain*	*per mg. N_2*
Stillborn	87·4	0·86	26	30	6,800	7,910
2 months	85·8	0·76	45	59	9,890	13,010
3 months	85·6	0·70	39	56	8,700	12,430
8 months	83·8	1·18	88	75	42,215	35,800
6 years	83·1	2·74	377	137	7,860	2,870

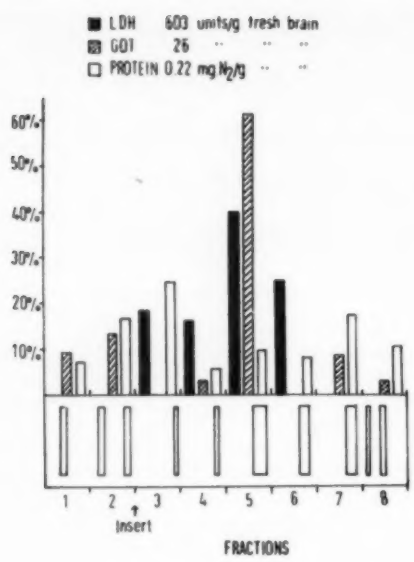

FIG. 5. *Normal white matter.*

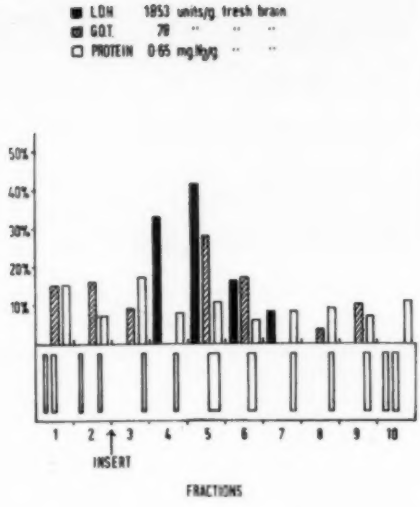

FIG. 6. *Normal cortex.*

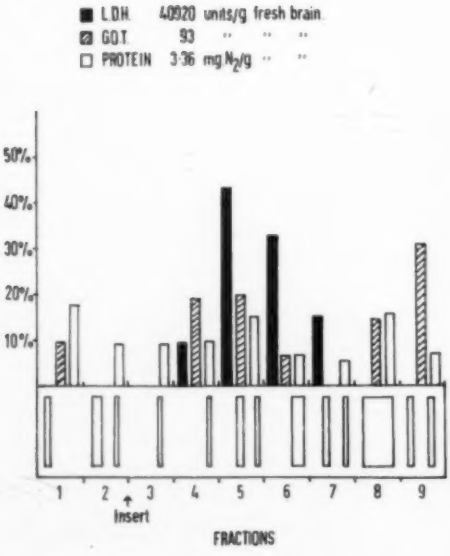

FIG. 7. *Oedematous cerebral white matter.*

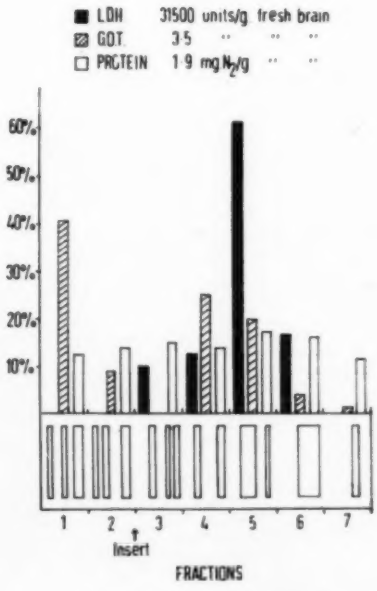

FIG. 8. *Oedematous cortex.*

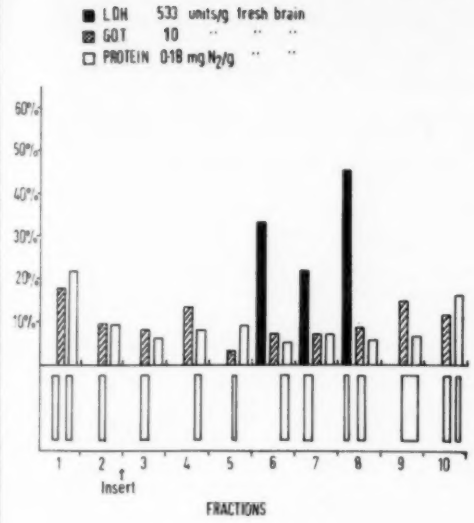

FIG. 9. *Infarcted cerebral white matter.*

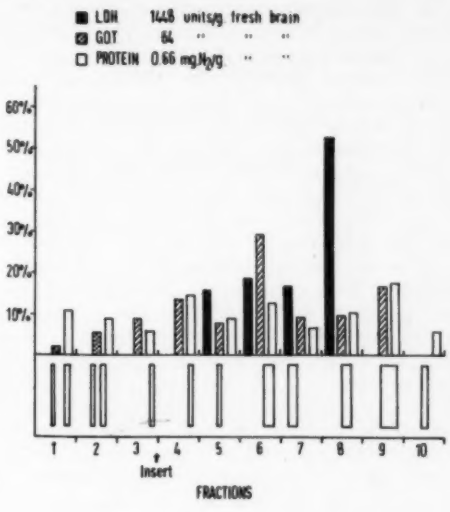

FIG. 10. *Infarcted cortex.*

were not well defined were found. Later other workers—Booij (1957), Kiyota (1959a), Tomonaga (1959)—found more fractions and these were frequently designated as being α, β, and γ globulin as well as albumin and pre-albumin. Recently Robertson (1960) by paper electrophoresis found one γ globulin, three α and three β globulins, one albumin, and two pre-albumin bands. He estimated the protein content of each fraction and reported more of fraction 4 in the cortex than in the white matter and more of fraction 7, probably an α globulin, in white than in grey matter. The results that have been found in starch would be in general agreement with these findings. Karcher *et al.* in two papers in 1959 examined these proteins by agar electrophoresis and they have described some seven to nine fractions in various areas and their results are very similar to those recorded here, but they did not obtain such a good separation of the fractions. Their method does, however, possess the advantage that a curve can be obtained by scanning the agar strip in a densitometer, whereas it has been necessary here to estimate the nitrogen content of the fractions separately. They found more pre-albumin and albumin than was found by the method recorded here. However, the degree of separation obtained on paper is not always very satisfactory.

Other organs besides the brain show soluble proteins, as Schwarzmann (1959) showed for the liver and stomach. He found up to 15 fractions for the former organ and 13 for the latter and his photographs do show some resemblance to those figured here.

The soluble protein content of the developing human brain has not been examined previously and more work is necessary before any useful opinions can be formulated. The lack of good differentiation between the different protein fractions found in these experiments was also observed by Palladin (1957) in his work on soluble proteins from the brains of cow embryos.

Among other reports concerning oedematous cerebral tissue are those of Kaps (1954) who found albumin in the white matter but not in the cortex, whereas half of the cases examined here did show the presence of albumin in the cortex. Tomonaga (1959) found some increase of protein in fractions 5 and 6 and Kiyota (1959b) also examined the soluble proteins from eight brains showing oedema. He described 'simple' and 'complicated' oedema, finding in the former a normal protein pattern, while in the latter abnormal protein fractions were present such as serum albumin in the brain extracts. The results recorded in this paper indicate a considerable amount of albumin to be present in the white matter in all cases and in the cortex in some cases. Further, although there was some increase in the L.D.H. content in the extracted fluid, no increase in G.O.T. was found. This may well suggest the possibility that both the water and albumin were extracellular

rather than intracellular and might therefore support the contention of Davson and Spaziani (1959) that such a space does exist. It is of interest that the soluble cerebral proteins from the infarcted brain, although containing albumin, yet did not yield a greater enzyme content than normal so that there may well be two types of oedema as Kiyota suggested.

When cerebral oedema takes place around a tumour or an abscess albumin appears to exude through the capillary walls, which may be damaged, into the extracellular space. Some of the albumin might be adsorbed on to the astrocytes with resulting damage to them or to their enzyme systems thereby causing rupture of some of these cells, so liberating L.D.H. but little G.O.T. This appears to be somewhat surprising, for McArdle, Thompson, and Webster (1960) have shown that lysolecithin liberated G.O.T. from incubated brain slices even though a very slight amount of true cholinesterase was liberated. At the present time it is not known exactly where in human nerve cells these enzymes are located but McArdle and his colleagues have demonstrated that in the rat's brain there is a considerable amount of G.O.T. in the heavy mitochondria.

Enzyme studies on proteins separated by starch electrophoresis have also been made by Hughes (1959; 1961) from normal and abnormal muscle. It is of interest to note that Wieme (1959) found a multiplicity of the L.D.H. group of enzymes in the tissues of the rat and mouse using agar electrophoresis. This appears to be true for the liver, kidney, and muscle, and it will be interesting to apply procedures similar to those of Wieme to the brain. Kowlessar, Pert, Haeffner, and Sleisenger (1959) have shown that serum alkaline phosphatase migrated in two bands when examined by starch electrophoresis. They also found that 5-nucleotidase was located in one serum protein fraction only. It has also been shown electrophoretically that there may be alterations in G.O.T. in the different protein fractions in diseases of different pathology (Emmrich and Zimmermann, 1959).

It is too early to speculate upon the value of these investigations in human disease but they may eventually yield a better understanding of the nature of the serious problem of brain oedema which is found in a variety of conditions.

My thanks are due to the many pathologists who have helped in this investigation by sending me brains for examination. Mr. R. Shortman has given me expert technical assistance and to him I am very grateful, as I am to Mr. J. A. Mills for his photographic skill.

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The usefulness of counting 'heat-affected' red cells as a guide to the risk of the later disappearance of red cells after burns

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SYNOPSIS The technique for counting heat-affected red cells ('microcytes') in the head of the blood film taken from patients on admission is described. Although crude, it does appear to increase the precision with which it is possible to predict the disappearance of red cells in the first 24 hours after a burn, and therefore to decide those cases in which ^{51}Cr red cell volume estimates may be clinically useful.

When a blood film is taken from a patient with a severe burn half an hour or more after injury, a small proportion of the red cells are very small. Occasionally 'budding' red cells are seen from which these small red cells are presumably derived. In the present communication these very small red cells have, when they are less than 3μ in diameter, been called 'microcytes'. These microcytes, which retain the smooth contour of a red cell, are often associated with red cell fragments and spherocytes. If an anaesthetized rat is given a full-thickness skin loss burn involving more than 10% of the body surface, similar microcytes, red cell fragments, and occasional spherocytes appear in the tail blood within five minutes. Similar findings can be produced by heating whole blood under suitable combinations of time and temperature in a test-tube. The presence of these abnormal cells, produced almost certainly by the direct effect of heat on the red cells in the area of the burn, has been described by Shen and Ham (1943) and Ham, Shen, Fleming, and Castle (1948).

The microcyte count on the blood film on admission on 134 patients has proved of clinical value as one of the signs used to predict the risk of red cells later disappearing (Jackson, 1959; Topley, Cason, Jackson, and Davies, 1961). For this reason the technique of counting microcytes is described and evidence presented on the association between the blood film on admission and the later disappearance of red cells measured by red cell volume estimates.

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TECHNIQUES

BLOOD FILM The aim is to count the proportion of red cells less than 3μ in diameter. Such cells can be more precisely counted than fragments, which merge imperceptibly into 'red cell dust', and than spherocytes, which merge imperceptibly into normal red cells. Microcytes appear in largest numbers in the head of the blood film immediately in front of the starting drop and decrease in numbers towards the tail. Most consistent counts of these cells have been obtained by confining the counting to fields close to the head and where red cells do not clump. The blood film was stained by a Romanowsky stain, usually Leishmann-Giemsa. At least 500 cells were counted, using a micrometer eye-piece and oil immersion objective of such a magnification that one division on the micrometer scale was equivalent to 1μ.

The depth of staining of the haemoglobin is consistent with these cells being very small red cells rounded off from larger red cells. The small cells are therefore very pale and can be missed if the staining is not efficient and the focus poor. With Romanowsky staining it was usually easy to differentiate 'microcytes' (less than 3μ) from platelets even though they can be of the same size. Small red cells and a platelet are shown in Fig. 1 and in the figures of Ham and his colleagues (1948).

Observer bias influences the decision to make new films or further films. It also influences the fields selected, and the red cells not counted because they were 'fragments' and not 'cells'. All the observations were made by one observer. The films from the first 68 cases were recounted using a numbering system by which the observer did not know which film was from which patient. Almost all the films from more recent cases have been counted within a few hours of admission before the later disappearance of red cells had been measured. Recounts on many of these cases have shown that the report 'less

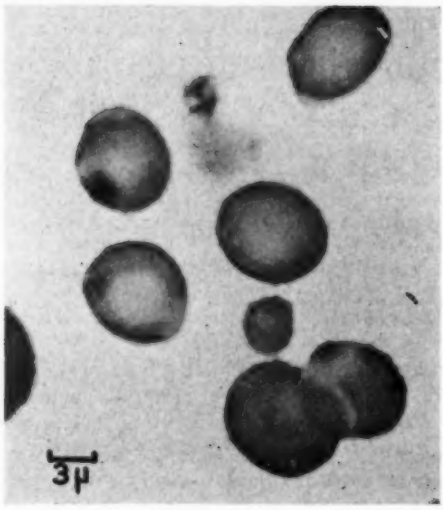

FIG. 1. *A typical microcyte in the blood film taken within four hours of burning injury.*

than 2% of microcytes' was almost invariably consistently obtained on recounts. When there were more than 2% of microcytes there was more inconsistency but the results almost invariably agreed to within ± 5%.

This technique carries all the disadvantages of differential counting of cells on spread blood films but no more satisfactory method has been found. The heat-affected red cells have not been separated satisfactorily by differential centrifugation or differential sedimentation in a variety of solutions.

RED CELL DISAPPEARANCE This has been measured by red cell volume estimates using ^{51}Cr- or ^{32}P-labelled red cells, combined with a knowledge of the patient's average normal red cell volume calculated from height and the volume of blood transfused. The method and calculation of the 'red cell volume estimate of red cell disappearance' has been described elsewhere (Davies and Topley, 1959). The results have been expressed as a percentage of the total circulating red cell volume. A 20% disappearance would therefore be equivalent to 0·3 litres of whole blood in a child of 6 years (115 cm. tall) and 1·0 litres of whole blood in an adult man (175 cm. tall).

AREA OF FULL-THICKNESS SKIN LOSS This was assessed by the clinical staff on admission, and checked in the light of subsequent grafting operations.

USE OF BLOOD FILM ON ADMISSION AS A GUIDE TO RED CELL DISAPPEARANCE DURING THE ENSUING DAY

The result of the blood film taken on admission has been compared with the red cell volume estimate of red cell disappearance 'about 24 hours' (12-36 hours) after admission. Because the area of full-thickness skin loss is itself a useful guide to disappearance of red cells the patients have been divided into four groups according to this area.

From 1954 to 1959 blood films on admission were taken from many patients with burns involving more than 20% of the body surface. The radioactive red cell volume estimate of red cell disappearance was used only when red cell disappearance might be clinically important. Table I summarizes the results of all blood films on admission, probably a fair sample of more than 15% full-thickness skin loss burns, but including a selection of the clinically more severe smaller burns. This table can be used for relating the blood film to the area of full-thickness skin loss. Table II summarizes the results on all patients who had a blood film on admission and a

TABLE I

RELATION OF BLOOD FILM FINDING TO AREA OF FULL-THICKNESS SKIN LOSS IN 134 PATIENTS

Area of Full-thickness Skin Loss	*Microcytes (%) in Blood Film on Admission*			*Total Patients*
	Less than 2	*2 to 9*	*10 or more*	
Less than 15%	24	11	0	35
15-29%	20	11	9	40
30-40%	5	20	6	31
More than 40%	3	16	9	28
Total patients	52	58	24	134

TABLE II

RELATION OF BLOOD FILM FINDING AND AREA OF FULL-THICKNESS SKIN LOSS TO RED CELL DISAPPEARANCE MEASURED BY RED CELL VOLUME ESTIMATE ABOUT 24 HOURS AFTER BURNING IN 55 PATIENTS

Microcytes in admission blood film	*Less than 2%*			*2-9%*			*10 or more %*			*Total Patients*
Red cell disappearance (%)	*<20*	*20+*	*Total*	*<20*	*20+*	*Total*	*<20*	*20+*	*Total*	
<15% full-thickness skin loss	6	0	6	4	1	5	0	0	0	11
15-29% full-thickness skin loss	6	0	6	2	2	4	0	4	4	14
30-40% full-thickness skin loss	1	0	1	5	6	11	1	4	5	17
>40% full-thickness skin loss	0	0	0	3	4	7	2	4	6	13
Total patients	13	0	13	14	13	27	3	12	15	55

red cell volume estimate after injury. This is not a fair sample of all shock cases because increasingly the presence of less than 2% of microcytes on the blood film was taken as one part of the evidence for predicting that red cells were unlikely to disappear and a radioactive chromium red cell volume estimate was therefore contraindicated. Table II can be used to relate blood film findings to red cell disappearance but must not be used to relate blood film findings to the area of the burn.

LESS THAN 2% OF MICROCYTES have never been associated with a more than 20% disappearance of red cells (0/13 patients, Table II). In less than 15% full-thickness skin loss burns less than a third of patients (Table I) had 2% or more of microcytes in their blood film; but such a finding when present was on one in five occasions associated with a more than 20% disappearance of red cells (Table II). Less than 2% of microcytes were found in half (20/40) the 15-29% full-thickness skin loss burns (Table I). In larger areas of full-thickness skin loss there were almost invariably larger numbers of microcytes. The finding of less than 2% of microcytes may therefore be added evidence that not more than 20% of red cells are going to disappear, rendering the prediction more precise than if the burn area alone were used. This added precision is of particular value in less than 30% full-thickness skin loss burns.

MORE THAN 10% OF MICROCYTES were not found in less than 15% full-thickness skin loss burns (Table I). When present in larger burns they were in 12 out of 15 patients associated with a more than 20% disappearance of red cells (Table II). The amount of measured disappearance ranged from 21 to 46% of the total circulating red cell volume.

DISCUSSION

As reported elsewhere (Jackson, 1959; Topley *et al.*, 1961), the area of full-thickness skin loss, the presence of haemoglobinuria 12-48 hours after injury, the amount of colloid transfused, and the microcytes in a blood film on admission are useful guides to predicting to the nearest 20% the likely disappearance of red cells during the first 24 hours after injury in more than 15% patients with full-thickness skin loss burns. All the results together are more precise than one result alone, and help to decide whether a ^{51}Cr red cell volume estimate is required to assess red cell loss more precisely. It is just in these cases, with more than 15% full-thickness skin loss, that mortality might be reduced during the shock stage by a nice appreciation of red cell and plasma losses, although in practice the mortality was not reduced over the period of this investigation (Bull, 1958). Such burns are a minority of the cases requiring colloid transfusion. The man-hours in the laboratory to count such films would be minimal. The main criticism of the method is the inherent inaccuracy of a technique based on differential counting in a selected area of a blood film.

The reason for finding an association between the blood film on admission and later disappearance of red cells is not clear. Although the possibility exists that a later haemolytic anaemia or haemorrhagic diathesis is triggered off by the initial heat effect on red cells and capillaries in the burn area, there are many other equally plausible explanations for this association.

I would like to thank Mr. D. MacG. Jackson and Mr. J. S. Cason for collaboration in this study on their patients, to acknowledge the assistance of the Burns Unit staff in the preparation of blood films, of Mrs. D. McKerracher in their staining, and of Dr. J. W. L. Davies and Miss Janet Frost and their colleagues in many of the red cell volume estimates.

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The distribution of blood cells on haemacytometer counting chambers with special reference to the amended British Standards Specification 748 (1958)

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SYNOPSIS The distribution of leucocytes and erythrocytes on the haemacytometer counting chamber is shown. Differences between total leucocyte counts on single and double-ruled chambers are given and the effect of different lengths of cover glass are discussed. An assessment of the relative accuracy of the amended British Standards Specification (B.S.S.) 748 (1958) double-ruled bright line chamber and the old B.S.S. chambers is made.

Hynes (1947) published a detailed account of the leucocyte distribution on the counting chamber, from which it was apparent that the distribution of cells departed considerably from the theoretical expectation that the number of cells on each square of a haemacytometer counting chamber is distributed in a Poisson series (Fig. 1).

Using a Burker single-ruled counting chamber with Neubauer ruling, Hynes counted, as is usual, the number of cells on four areas of one square millimetre at the corners of the ruled area and recorded the four subtotals separately. Over this series of counts the two squares nearest to the filling point of the chamber gave nearly identical totals 3·5% below the mean value for all four squares, and the two further squares similarly gave nearly identical totals 3·5% above the mean value.

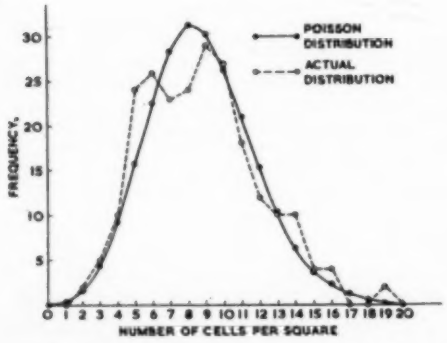

FIG. 1. *Comparison of theoretical with actual distribution of leucocytes counted in squares 0·5 mm. × 0·5 mm. over the whole flooded area.*

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By counting known areas across the length of the chamber Hynes demonstrated that there was a variation in leucocyte density along the length of the counting chamber, and that the density increased progressively from the point of entry of the fluid. The curve of the leucocyte density distribution along the flooded area had two maxima and could be expressed statistically by a quartic polynomial with the mean value for the leucocyte density per unit area being reached at the centre of the chamber. Apparently, the position of the ruled area of the chamber did not affect the slope of the leucocyte distribution curve, and the distribution of cells across a narrow segment of the area conformed to a Poisson expectation. Furthermore, the variation in the leucocyte distribution was not due to bending of the cover glass, nor to any change in the cell distribution in the pipette before the chamber was filled. It appeared to be due to the drift of the leucocytes caused by their initial forward impetus and the subsequent convection currents. The distribution of red cells on the counting chamber followed similar laws to those of the leucocytes.

Hynes found, and it has been confirmed at A.E.R.E. (Sanders, 1954), that the leucocyte and red

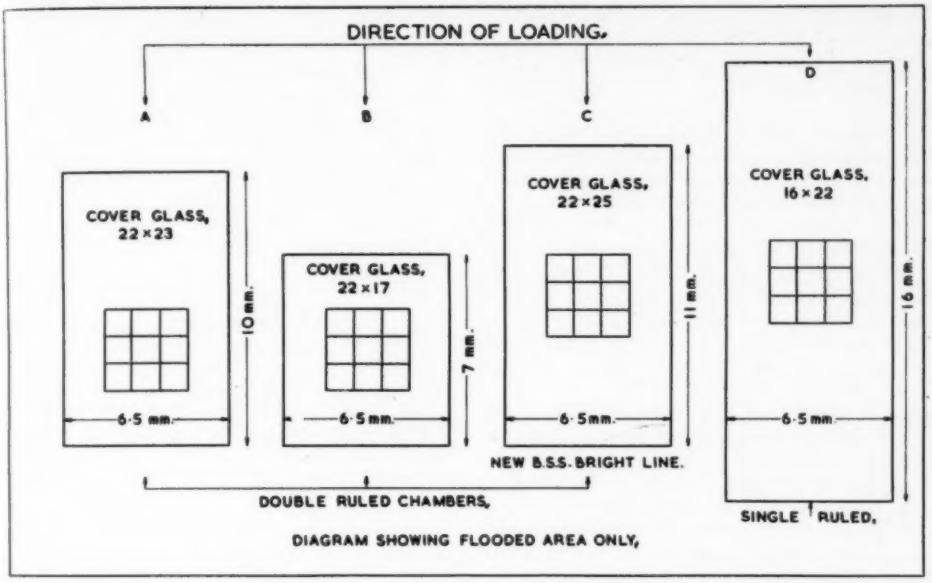

FIG. 2

A *The actual flooded area on the old B.S.S. double-ruled chamber with the ruled area in the incorrect position.*
B *The same chamber with the ruled area in the centre of the flooded area using a 17 mm. × 22 mm. cover glass.*
C *The amended B.S.S. (1958) metallized bright line double-ruled chamber.*
D *A single-ruled chamber with 16 mm. × 22 mm. cover glass.*

cell distribution on double-ruled counting chambers (with the central trough and two ruled areas) varies in a similar fashion. Again the rule holds that the leucocyte or red cell density reaches its mean half-way along the flooded area. It was clear from this work that the true count is only obtained if cells are counted in equal areas symmetrically disposed about the centre of the flooded area.

Using a single-ruled counting chamber this can easily be achieved if sufficient care is taken to place the cover glass in the correct position, but with the double-ruled counting chamber it is more difficult to ensure that both ruled areas are centrally placed with regard to their respective flooded areas beneath the cover glass. In fact, with the specification and size of cover glass stated in B.S.S. 748 (1937-53), it is impossible to use both sides of the counting chamber at the same time and have each ruling centrally placed with regard to its flooded area. This specification states that the size of cover glass to be used with the double-ruled chamber shall be 23 mm. × 22 mm., and that the distance between the centres of the two ruled areas shall be 10 mm. With this size of cover glass symmetrically placed in relation to the central dividing trough, the ruled area of each side is not centrally placed with regard to its flooded area. The mid-line of each ruled area will be 3·5 mm. from the central dividing trough and 6·5 mm. from the outer edge of the cover glass or point of entry of the diluted blood sample (Fig. 2a). As the leucocyte or red cell density reaches its mean value half-way along the flooded area of the chamber the use of this type of counting chamber would result in cell counts being higher by approximately 7·5%. To correct this error it would be necessary to reduce the size of the cover glass to 17 mm. × 22 mm. (Fig. 2b). However, this would reduce the length of the flooded area to 7 mm.

Hynes has shown that the length of the flooded area has a considerable effect on the slope of the distribution curve. The curve has a steep gradient in the middle third of the filled area, but the steepness decreases as the length of the counting chamber filled increases. With different lengths of flooded area, however, the curves show one important common property, namely, that the cell density reaches its mean value half-way along this area. Using the double-ruled chamber, the position of

the ruled area would have to be altered and if possible the length of the chamber increased since the longer the area the less would be the error from slight inaccuracies in the position of the rulings.

The error in position of the ruled area and the size of the cover glass was brought to the notice of the appropriate committee of the British Standards Institution, who in 1958 issued amendment No. 2 for B.S.S. 748 in respect of double-ruled counting chambers. These chambers are now made in Great Britain with the distance between the centres of the ruled areas 14 mm. and with a cover glass of 25 mm. × 22 mm. This size of cover glass provides a flooded area of 11 mm. in length on each side of the chamber, and ensures that the rulings are centrally placed.

The introduction of the 'metallized bright line' type of chamber has greatly increased the clarity of the rulings, and for cell counts this type of chamber is far superior to the old type of haemacytometer. It is of interest to note that after over two years of daily use these chambers have shown no deterioration in the metallized surface, whereas the rulings on the old type of chamber became faint even after a few months' use.

When the new haemacytometers became available, it was decided to carry out a further series of experiments on the distribution of cells and the relative accuracy of the new and old type of counting chambers.

METHODS

Blood samples, either venous or capillary, were diluted 1 in 20 for leucocytes with 2% acetic acid tinted with crystal violet, by means of haemacytometer dilution pipettes, each of which had been checked by the National Physical Laboratory. Only those pipettes giving an exact dilution of 1 in 20 were used. The red cells were diluted 1 in 200 by means of accurate 0·02 ml. straight line pipettes, washing the contents into exactly 4·0 ml. Wolff's fluid contained in 5 ml. plastic stoppered tubes (potassium iodide 26·0 g., iodine 1·0 g. made up to 1,000 ml. with distilled water to which is added 30·0 ml. of 1% aqueous solution of eosin). The contents of the haemacytometer pipettes were thoroughly mixed by placing the pipettes in a magnetic shaker (Sanders, Parry, Bennett, and Geison, 1950) for three minutes. The first few drops of the mixed contents of each pipette were expelled before loading the chambers in the usual way by capillary attraction. The red cell suspensions were mixed by placing the tubes in a rotary shaker for five minutes and a sample of the contents removed by means of a Pasteur pipette. The chambers were loaded as previously stated. Preparations were sealed with vaseline to prevent drying out when counting cells for distribution across the length of the flooded area.

PROBLEM 1*a* Was the distribution of cells along the length of the flooded area similar to that described by Hynes?

A double-ruled improved Neubauer chamber (old B.S.S., 1953) was used for this set of experiments (1) with a cover glass of 17 mm. × 22 mm. providing a flooded area of 7 mm. in length and with the rulings centrally placed; and (2) with a cover glass of 22 mm. × 23 mm. providing a flooded area of 10 mm. in length with the rulings in the incorrect position.

Experiment 1 Distribution of leucocytes on the double-ruled chamber, flooded area 10 mm. in length (Fig. 2a).

Areas of 0·5 mm. square on the counting chamber were defined by means of Ehrlich's eyepieces, and counts were made starting 0·5 mm. from the point of entry of the diluted blood sample across the length of the chamber to within 0·5 mm. of the dividing trough. Twelve such rows of counts were necessary to provide a grid of observations covering the whole of the flooded area. Five separate loadings were made from the same blood sample, and the mean for all five counts in each position of the grid plotted as shown in Fig. 3. It will be seen that

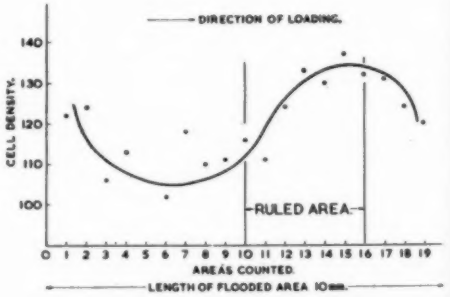

FIG. 3. *The leucocyte distribution on the double-ruled chamber, old B.S.S. (1953), flooded area 10 mm. in length, with the ruled area in the incorrect position. The curves are the best fitting quartics; the dots show the observed values relating to the continuous lines.*

the mean distribution of cells is in the centre of the flooded area, but that using this counting chamber with a cover glass of 22 mm. × 23 mm., the ruled area is situated in the higher concentration of cells.

Experiment 2 Distribution of leucocytes on the double-ruled chamber, flooded area 7 mm. in length (Fig. 2b).

Experiment 1 was repeated using the shorter flooded area and the mean results plotted as before (Fig. 4). The mean concentration of cells is again in the centre of the flooded area, but due to the reduction in size of the cover glass the rulings are centrally placed.

Experiment 3 Distribution of red cells on the double-ruled chamber, flooded area 7 mm. in length (Fig. 2b).

This experiment was similar to experiment 2, but in this case red cells diluted 1 in 200 were used. Five

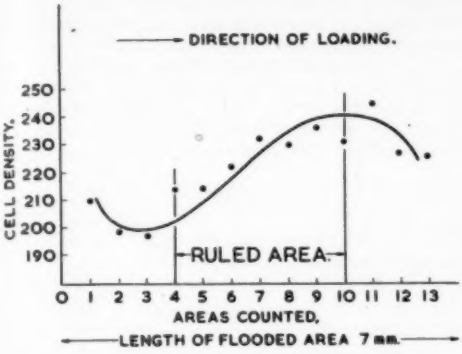

FIG. 4. *The leucocyte distribution on the double-ruled chamber, old B.S.S. (1953), flooded area 7 mm. in length, with the ruled area in the correct position. The curves are the best fitting quartics; the dots show the observed values relating to the continuous line.*

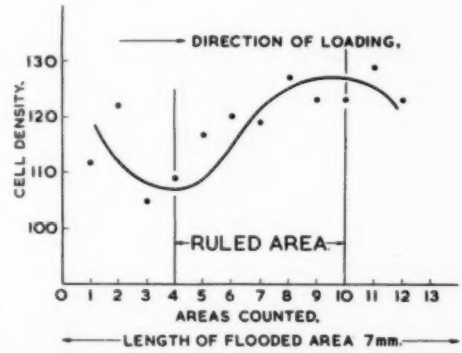

FIG. 5. *The red cell distribution on the double-ruled chamber, B.S.S. (1953), flooded area 7 mm. in length, with the ruled area in the correct position. The curves are the best fitting quartics; the dots show the observed values relating to the continuous line.*

separate loadings were made from the same sample as in the previous experiments and the mean results plotted (Fig. 5). A similar type of distribution curve to that of the leucocytes was obtained.

PROBLEM 1*b* Does the length of the flooded area have any effect upon the distribution of the cells?

The length of the flooded area affects the slope of the distribution in that the longer the area the less the slope of the curve. Irrespective of the length of the flooded area the mean value for the cell density is reached half-way along the area.

PROBLEM 2*a* What is the difference between the total leucocyte count made on a single-ruled chamber and that made on the double-ruled chamber with the ruled area in the incorrect position?

Experiment 1 Blood samples were diluted 1 in 20, and mixed as previously described, and the contents of each pipette loaded on to both sides of a double-ruled chamber, old B.S.S. (1953), improved Neubauer, with a cover glass of 22 mm. × 23 mm., flooded area 10 mm. in length; and twice on to a single-ruled chamber with a cover glass of 22 mm. × 16 mm., flooded area 16 mm. in length. Three trained technicians carried out the counts using 50 separate blood samples, *i.e.*, 100 counts on each chamber. The cells were counted in the four corner square millimetre areas. The results are given in Table I as the mean of the 50 blood samples.

TABLE I

TOTAL LEUCOCYTE COUNT

Technician	*Single-ruled Chamber*	*Double-ruled Chamber*	*Difference between Two Chambers (%)*
A	6,050	6,720	11·07
B	6,440	7,340	14·00
C	6,210	7,035	13·30
Mean	6,266	7,032	12·79

The difference of 12·79% between the results of the counts made on the single-ruled chamber and the double-ruled chamber is greater than was expected. The incorrect position of the rulings in the old B.S.S. double-ruled chamber could give rise to a count higher by approximately 7·5% than those obtained when the rulings are in the correct position. The additional 5·29% difference suggests that the longer flooded area in the single-ruled chamber has an effect on the total cell count result.

PROBLEM 2*b* What is the difference between the total leucocyte count made on a single-ruled chamber and that made on the amended B.S.S. double-ruled chamber.

Experiment 2 Twenty-two blood samples were loaded on to both sides of the double-ruled chamber with a cover glass of 25 mm. × 22 mm., flooded area 11 mm. in length, and twice on a single-ruled chamber with a cover glass of 22 mm. × 16 mm., flooded area 16 mm. in length. One trained technician carried out the counts. The results are given in Table II, as the mean for all the blood samples.

TABLE II

TOTAL LEUCOCYTE COUNT

Single-ruled Chamber	*Double-ruled Chamber*	*Difference between Two Chambers (%)*
6,300	6,650	5·6

These results show that although the length of the flooded area is 11 mm. on the double-ruled chamber with the rulings centrally placed, there is still a difference of 5·6% between the counts made on the two chambers.

This difference is similar to that obtained in experiment

1 but since both ruled areas in the single and double-ruled chambers were centrally placed with regard to the flooded area, this difference cannot be due to incorrect positions of the rulings but due to the difference in length of the flooded area.

PROBLEM 2*c* What is the difference between the total leucocyte count made on the amended B.S.S. double-ruled chamber and that made on the old B.S.S. double-ruled chamber with the ruled area in the incorrect position?

Experiment 3 Three trained technicians carried out the counts, each using 50 separate blood samples diluted 1 in 20, loading both sides of each chamber. The results are given in Table III as the mean of the 50 blood samples.

TABLE III

TOTAL LEUCOCYTE COUNT

Technician	*Amended B.S.S. Double-ruled Chamber*	*Old B.S.S. Double-ruled Chamber*	*Difference between Two Chambers (%)*
A	7,210	7,715	7·0
B	5,215	5,620	7·8
C	6,770	7,309	8·0
Mean	6,398	6,881	7·6

$t = 6{\cdot}3$ (50 blood samples) P = less than 0·01

The results show a mean of 7·6% difference between the two types of chamber. Applying the t test to the results of technician A, the probability of the difference between the two means occurring by chance alone is less than one in a hundred. Therefore the difference is unlikely to be due to chance but due to the incorrect position of the ruled area on the old type of chamber.

PROBLEM 3 What is the difference in the total leucocyte count when made using different lengths of flooded area and with the ruled area off centre by 1 and 2 millimetres?

Experiment To compare the error due to different lengths of flooded area, and that due to incorrect positioning of rulings, a series of leucocyte counts was made using three different sizes of cover glass 11, 14, and 17 mm. in length on a single-ruled chamber. For each size of cover glass a count was made with the ruled area centrally placed in relation to its flooded area, and also with the cover glass so placed that the rulings would be 1 mm. and 2 mm. off centre, *i.e.*, so that the ruled area would be in the more concentrated cell density. The results are given in Table IV, as the mean of five blood samples.

TABLE IV

Source of Variation	*Coefficient of Variation*
Between the three lengths of flooded area 11, 14, and 17 mm.	8·8%
Between the three positions of the ruled area; central, 1 and 2 mm. off centre	16·4%
Random error	12·4%

The results show that the variation is greater (16·4%) when the rulings are incorrectly positioned in relation to the flooded area than it is with different lengths of flooded area (8·8%). Although Hynes has shown that the longer the flooded area the less is the error from slight inaccuracies in the position of the ruling, he has not considered the length of the flooded area as being a factor in the variation between cell counts.

PROBLEM 4 How far off centre are the ruled areas in a selection of the old type of double-ruled chambers and a selection of the new amended B.S.S. double-ruled chamber?

Four types of double-ruled chambers—Burker, ordinary Neubauer, improved Neubauer (1937-53), B.S. specification, and the amended B.S.S. 748 (1958) were examined. The distance between the edge of the central dividing trough and the beginning of the 3 mm. × 3 mm. ruled area was measured to within 0·1 mm. by means of a Zeiss photomicroscope. The results are given in Table V to the nearest 0·2 mm.

Of the 20 amended B.S.S. double-ruled chambers measured, the maximum difference between the actual and specified position of the ruled area was 0·2 mm. The effect of this small difference on the cell count is negligible.

With the old B.S. specification the ruled area of

TABLE V

Actual Measurement (mm.)	*Difference between B.S.S. and Measurement (mm.)*	*Type of Chamber*			*% Higher than Mean Count of Correct Chamber*
		Burker	*Ordinary Neubauer*	*Improved Neubauer*	
1·2	+0·8	1	0	0	13·0
1·4	+0·6	3	0	0	11·0
1·6	+0·4	2	2	7	
1·8	+0·2	1	8	10	
2·0	0·0	1	20	9	6·0
2·2	−0·2	6	9	8	
2·4	−0·4	1	1	6	4·0
2·6	−0·6	0	0	0	
2·8	−0·8	0	0	0	
3·0	−1·0	0	0	0	
3·2	−1·2	0	0	0	
3·4	−1·4	1	0	0	0·0
No. of ruled areas examined		16	40	40	

3 mm. × 3 mm. should start at 2·0 mm. from the dividing trough and with the new specification 4·0 mm. The results show that apart from the fact that the position of the rulings on the old chambers differ from the specification, there is also considerable variation among a selection of the same type of chamber.

DISCUSSION

Hynes (1947) has shown, and it has been confirmed in this department, that the density of cells per unit area on the counting chamber increases progressively from the point of entry of the fluid along the length of the flooded area; that the mean cell density per unit area is reached at the centre of the flooded area of the chamber and that the rate of change in the cell density is less the longer the filled area of the counting chamber, in other words, the less the slope of the cell distribution curve.

In order to obtain accurate counts it is essential to count the cells in equal areas symmetrically disposed about the centre of the flooded area. Therefore it is important that with double-ruled chambers the position of the rulings and the size of cover glass must be clearly specified so that the haemacytometer will be manufactured to within accurate limits. Measurements made on the amended B.S.S. double-ruled chambers have shown that these tolerances are being adhered to.

Hynes found that with a flooded area of 9·0 mm. in length, the leucocyte density per unit area changed at the rate of 5% per mm. at the centre, whereas with a flooded area of 26·0 mm. in length the rate of change was only 2% per mm. Thus the longer the area of the counting chamber filled, the less would be the error from small inaccuracies in placing the cover glass centrally over the rulings.

With the double-ruled chambers the length of the flooded area on each side of the chamber is restricted by the overall dimensions of the haemacytometer. Allowance must be made for a small plateau at the outer edge of each side of the chamber in order to load the diluted blood sample by capillary attraction. The cover glass cannot therefore be increased in size beyond 25·0 mm. in length. This size of cover glass allows for a flooded area of 11·0 mm. on each side of the chamber with 3·0 mm. to cover the dividing trough between the two rulings.

It has been shown that counts made from the same blood sample on the amended B.S.S. double-ruled chamber give results of 5·6% higher when compared with those made on a single-ruled chamber with a flooded area of 16·0 mm. in length; and that when made on the old B.S.S. (1953) improved Neubauer double-ruled chamber with the rulings in the incorrect position, and a single-ruled chamber the difference was of the order of 12·79%.

Although the mean cell density is reached at the centre of the flooded area irrespective of its length, there does appear to be an effect on the cell count depending upon the length of the flooded area. Little consideration appears to have been given to the fact that irrespective of whether it is a single-ruled or double-ruled chamber there are two constant dimensions which are common to both haemacytometers, the depth of the chamber 0·1 mm. and the width of the flooded area 6·0 mm. It is our opinion that there is probably an optimum length of flooded area, taking into consideration these two fixed dimensions, and that approximately twice the width of the flooded area would be a reasonable length for good loading and distribution of cells by the capillary attraction method. It is more difficult to load correctly, without streaming, a long narrow segment by capillary attraction.

A selection of the amended B.S.S. double-ruled chamber examined by the National Physical Laboratory with regard to accuracy of depth and other parameters showed that they are now being manufactured to within the tolerance stated by the British Standards Institution. It is our opinion that providing the cover glass is centrally placed on the chamber and due care is taken when loading the blood sample, accurate counts can be obtained with this haemacytometer.

CONCLUSIONS

Use of the old B.S.S. double-ruled chamber with the rulings in the incorrect position gives rise to cell counts higher by approximately 7·6% than those obtained using the amended B.S.S. double-ruled chamber with the rulings in the correct position.

Cell counts made on a single-ruled chamber with a flooded area of 16 mm. in length are approximately 5·6% lower than those made on the amended B.S.S. double-ruled chamber with a flooded area of 11 mm. in length.

It appears that there must be an optimum length of flooded area for accurate cell counts on haemacytometer counting chambers when loading the diluted blood sample by the capillary attraction method. Taking into consideration the two constant dimensions, the width and depth of the flooded area, we feel that approximately twice the width is the optimum length.

The amended B.S.S. double-ruled bright-line chamber is now manufactured in Britain to a high degree of accuracy. Provided that care in positioning the cover glass and loading diluted blood sample is taken reliable cell counts can be obtained using the haemacytometer.

SUMMARY

The density of cells per unit area on the counting chamber increases progressively from the point of entrance of the sample along the length of the chamber.

Irrespective of the length of the flooded area the cell density reaches its mean half-way along the chamber. The longer the flooded area the less is the slope of the distribution curve.

The results of total leucocyte counts made on single-ruled and double-ruled chambers show that the increased length of flooded area on the former gives rise to a lower count.

Measurements on a selection of the old type of double-ruled chambers show that the position of the ruled areas varies considerably from chamber to chamber but that the rulings on the amended B.S.S double-ruled chambers is accurate to within the British Standards specification.

It is suggested that there is an optimum length of flooded area for a standard width and depth of chamber when loading by the capillary attraction method.

We are indebted to Mr. B. D. Cook and Mr. D. J. Cotchin for performing many of the counts, to other members of the Haematology Department for their help, to Dr. R. G. Orr for much helpful criticism in the preparation of this paper, and to Dr. Katharine Williams, Principal Medical Officer, for her permission to publish the paper.

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J. clin. Path. (1961), **14**, 305.

The defect of blood coagulation in joints

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SYNOPSIS Clinical and experimental observations are described that indicate that intra-articular fibrinogenolysis is a cause of the defect of coagulation seen when blood is extravasated into a synovial joint.

The observation that blood shed into synovial joints apparently fails to clot has aroused interest for many years (Jaffe, 1897; Julliard, 1902; Kling, 1938; Ropes and Bauer, 1953). Studies on the behaviour of intra-articular fractures (Harrold, 1960a) have suggested that the fluid state of extravasated blood in joints may be one reason for the preservation of the joint space after injury and for the perpetuation of the fracture gap in intra-articular fractures. On this account a study of haemarthroses was undertaken, with particular reference to the factors that interfere with the clotting of blood in joints.

CLINICAL OBSERVATIONS

Although in clinical experience fluid blood may be obtained by aspiration or seen to flow out of the joint at operation, intra-articular clots are often found. Twenty-five traumatic haemarthroses were carefully inspected at operation. The findings are set out in Table I. Small soft clots were found in nearly half the cases. They were seen floating free or attached to injured surfaces and they did not adhere to healthy synovial membrane or to cartilage. Joints opened later than two weeks from injury were usually quite 'clean', with no trace of coagulated blood. The defect of blood coagulation was thus frequently incomplete.

TABLE I

CLINICAL HAEMARTHROSES

Duration of haemarthrosis	*<24 hr.*	*1-2 days*	*3-7 days*	*> 7 days*
Number of haemarthroses	9	5	5	6
No clot found	5	3	2	5
Small clots found	3	1	2	1
Over a quarter volume clotted	1	1	1	0
Over half volume clotted	0	0	0	0

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EXAMINATION OF HAEMARTHROSIS FLUIDS

The fluid component of seven human haemarthroses was examined. The haemoglobin concentration varied from 7·3 to 18·8 g./100 ml., and averaged 11·0 g./100 ml. All specimens contained mucin, none contained fibrinogen, and considerable haemolysis was seen in all. An antithrombin titration, by the method of Biggs and Macfarlane (1957), was performed on two specimens; no excess of antithrombin compared with normal human plasma was demonstrated. These specimens were also tested for free protease on a heated bovine fibrin plate (Astrup and Müllertz, 1952), with negative results.

The fluid component of a rabbit haemarthrosis, induced by injecting blood two hours before, was also examined. The haemoglobin concentration was identical with that of the heart blood injected, namely, 10·3 g./100 ml. Slight haemolysis was noted. The fibrinogen level was 2·3 mg./100 ml., that of the plasma being 263 mg./100 ml. There was no excess antithrombin and no free protease.

The fact that there was no excess antithrombin and no fibrinogen suggested that it was improbable that blood in joints failed to clot because of interference with the generation of thrombin or its action. These observations were, however, compatible with either blood coagulation followed within three or four hours by clot lysis, or with destruction of fibrinogen before clotting could occur.

EVIDENCE AGAINST RAPID CLOT LYSIS

A series of experiments in dogs tested the possibility that the fluid state of haemarthroses was because of the blood clotting followed by rapid clot lysis.

Mongrel dogs, 25-30 lb. (11·3-13·6 kg.) in weight and not more than 3 years old, were anaesthetized with Nembutal. Haemarthroses were induced by injecting 1·0 to 10·0 ml. freshly drawn venous blood

into the knee and shoulder joints, and by passing a 3 mm. drill across the elbow joints. Siliconed or paraffined all-glass syringes with size 12 needles were used. The joints were opened at intervals and the state of the contents determined by inspection.

In eight haemarthroses caused by drilling across the joint, and explored five, 10, 20, 40, 60 minutes and two, three, and four hours later, bloody fluid with no clot was discovered. In 17 haemarthroses produced by injecting blood, and explored 10, 15, 20, 30, 40, 60 minutes, and five and six hours later, partial clotting was observed in 14. The joints in which no clot formed were three of five that received only 1·0 ml. blood. There was no relationship between the quantity of clot found and the time since the haemarthrosis started and therefore no suggestion that clot lysis was occurring during the period of observation.

FIBRINOGENOLYSIS IN JOINTS

The evidence for fibrinogenolysis in synovial joints has already been described (Harrold, 1960b). When citrated plasma was injected into the knee of an anaesthetized rabbit, 3 to 4 months old, serial estimations showed a fall of fibrinogen level to zero within one and a quarter hours, a fall which was appreciably slower in fully grown rabbits (Fig. 1).

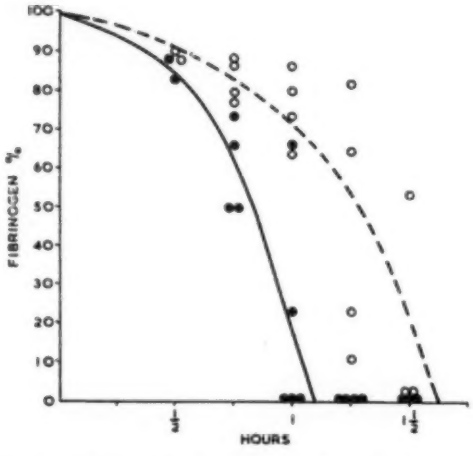

FIG. 1. *Fibrinogen levels of citrated plasma in the rabbit knee. The fibrinogen concentration is expressed as a percentage of the maximum. The solid dots, averaged by a continuous line, represent the results of five experiments in rabbits less than 8 months old. The open dots, averaged by a broken line, are derived from four experiments in rabbits over 11 months old.*

It was not accompanied by the formation of an equivalent amount of fibrin clot. Fibrinogenolysis was not seen in the recently dead animal. It was inhibited by heparin, 5 units/ml., and by soy bean trypsin inhibitor, 1·0 mg./ml.

A quantitative study of blood clot formation in joints was carried out to see if there were any correlation between the features of fibrinogenolysis and the defect of blood clotting in joints.

Preliminary experiments *in vitro* were necessary.

FACTORS INFLUENCING BLOOD CLOT WEIGHT IN VITRO Rabbit blood was collected by heart puncture into a 10 ml. siliconed syringe, a two-syringe technique being used. One millilitre aliquots were immediately measured from the syringe into a series of 1·0 × 7·5 cm. test-tubes in a water-bath at 37°C. After a set period the clot was gently dislodged from the sides of each tube, washed out with 2·0 ml. normal saline and filtered through a single layer of cotton gauze in a conical funnel. Filtration was for 15 minutes under cover to limit evaporation. The clot was then gently lifted off with forceps and weighed.

EFFECT OF TIME ON CLOT WEIGHT IN VITRO When rabbit blood was allowed to clot in plain glass tubes the weight of clot obtained did not reach a steady maximum until about two hours had passed (Fig. 2). Furthermore, the filtrates obtained in the first three-quarters of an hour themselves clotted. Thus, although the blood had apparently clotted within 10 minutes, a much longer period was needed for the process to go to completion. Similar findings were obtained when the experiment was repeated with human blood.

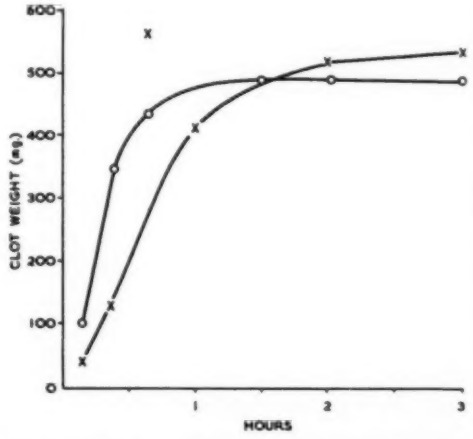

FIG. 2. *Weights of clot obtained from 1·0 ml. blood clotting in plain glass tubes at 37° C. Rabbit blood* (o——o) *and human blood* (×——×) *were used. The exceptional third clot weight in the experiment with human blood may be attributed to contamination of the test tube.*

EFFECT OF SPEED OF COAGULATION ON CLOT WEIGHT IN VITRO Standardized human brain thromboplastin giving a one-stage prothrombin time on normal human plasma of 15 to 20 seconds was prepared, and 0·1 ml. placed in the test-tube before adding the blood. A large weight of clot rapidly formed, decreasing to a steady level over the following hour. The final clot weight was greater, in four experiments, by an average of 18% over that obtained when no thromboplastin was added. Conversely, if clotting were delayed by placing the blood in siliconed tubes, the final clot weight was less by 15%.

EFFECTS OF DILUTION OF BLOOD ON CLOT WEIGHT IN VITRO When rabbit blood was diluted with saline, serum, or plasma the final clot weight obtained from 1·0 ml. diluted blood was directly proportional to the red cell content of the mixture. Diluting the blood with a suspension of red cells in saline had little effect on the clot weight up to 50% dilution but had an increasing effect after this (Fig. 3).

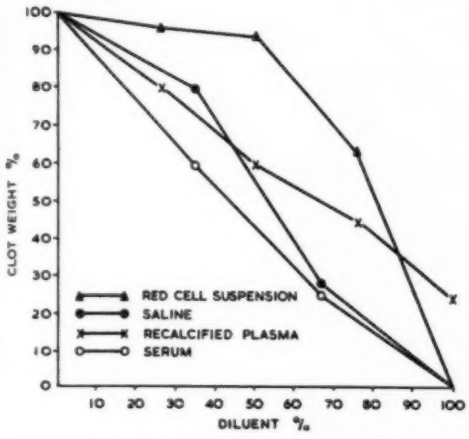

FIG. 3. *Effect of dilution on weights of blood clot.*

CLOTTING DEFECT IN THE RABBIT KNEE JOINT

With the rabbit anaesthetized with Nembutal, 1·0 ml. heart blood was withdrawn into a paraffined, all-glass Mantoux syringe and immediately injected through a size 20 needle already placed in the knee. A control *in vitro* was set up at the same time, using plain glass tubes without added thromboplastin. After two hours the animal was killed and the joint explored. Clot was gently removed with forceps and the joint washed out with 2·0 ml. saline. Filtration and clot weighing in the standard manner followed. The clot, filtrate, and filter gauze were then mechanically mixed and broken up. Distilled water was added to 10·0 ml. and the haemoglobin concentration of the supernatant estimated. From this the total haemoglobin in the joint was estimated. Knowing the amount of haemoglobin in 1·0 ml. heart blood, the clot weight obtained was corrected to the weight per millilitre of blood, thus allowing for either the effect of further bleeding into the joint, or more often, for leakage of blood out of the joint along the needle track and down the tibialis anterior tendon sheath.

The results in eight experiments in rabbits 3 to 4 months old are given in Table II. On average the corrected joint clot weights were 60% of those of the controls *in vitro*.

TABLE II

DEFECT OF CLOT WEIGHT IN JOINTS

Experiment	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Joint clot (mg.)	118	280	190	280	250	250	170	260
Joint Hb (mg.)	88	117	130	113	103	103	106	121
Blood Hb (mg./ml.)	112	117	163	112	126	123	124	148
Corrected joint clot (mg.)	150	280	235	280	306	300	200	317
Control *in vitro* (mg.)	260	490	510	405	485	415	395	490
Joint clot × 100 / control	57	57	46	69	63	72	51	65

Average correction applied to joint clot weight = 16·5% increase
Corrected joint clot weight averaged 60·0% control

CORRELATION OF JOINT FIBRINOGENOLYSIS WITH CLOTTING DEFECT

Fibrinogenolysis occurred more slowly in old rabbits than young. The corrected joint clot weights in three rabbits 14, 7, and 10 months old were 66, 89, and 97% of the controls respectively, averaging 84%.

Fibrinogenolysis takes time, and if clotting is accelerated by adding thromboplastin, the effect of fibrinogenolysis on clot weight should be much reduced. The weights were compared of clot obtained from 1·0 ml. blood *in vitro* and in a joint when 0·1 ml. standardized thromboplastin had been added to the tube and to the joint immediately before the blood. In four experiments the corrected joint clot weights were 75, 82, 98, and 96% of the controls, averaging 88%.

Fibrinogenolysis does not occur after death. One millilitre of blood was injected into one knee of an anaesthetized rabbit. After two hours the joint was opened and the clot collected in the usual way. Just before arthrotomy 1·0 ml. blood was injected into the opposite knee and the animal immediately killed. Body temperature was maintained in the hot room at 37°C. After a further two hours the second knee was opened and the clot extracted and weighed. The joint clot weights were compared with those of controls *in vitro*. In four experiments the dead joint clot weights were 81, 120, 64, and 96% of the controls, averaging 90%. The living joint clot weights were 58, 68, 48, and 65% respectively (average 60%).

DISCUSSION

These experiments support the view that fibrinogenolysis is responsible for at least part of the defect of clot formation noted when blood coagulates in a

synovial joint. Fibrinogenolysis takes at least one hour to go to completion, but the conversion of fibrinogen to fibrin in the absence of added thromboplastin has also been shown to be a relatively slow process.

It is possible that, as a result of fibrinogenolysis, secondary anticoagulant factors appear (Stormorken, 1957; Triantaphyllopoulos, 1958; Niewiarowski, Latallo, and Stachurska, 1959) which, by delaying clotting, favour more fibrinogenolysis. These factors are, however, labile and disappear with further proteolysis.

The presence of plasmin activator in joint tissues (Astrup and Sjølin, 1958; Lack, 1959), the inhibitory effect of heparin and soy bean inhibitor, and the observation that fibrinogenolysis is slower in older rabbits than in the young, are all compatible with fibrinogenolysis being caused by activation of the plasmin system.

When blood is shed into a synovial joint the plasma fibrinogen becomes the substrate for two competing reactions, namely, conversion to fibrin by thrombin or fibrinogenolysis, probably by plasmin. The balance between these reactions is probably affected by the age and species of the subject, the severity of the joint injury and hence the concentration of thromboplastin liberated, and by the speed of entry of the blood, a slow ooze probably favouring fibrinogenolysis by allowing time for secondary anticoagulant factors to appear. The absence of thromboplastin in normal human joint tissues (Astrup and Sjølin, 1958) also favours fibrinogenolysis rather than blood clotting.

The bulk of blood clot formed in a joint may later be diminished by the lysis of red cells that seems to be a constant feature of haemarthroses.

The cost of the experimental work on dogs was defrayed by a grant from the Joint Standing Research Committee, St. Mary's Hospital and Medical School.

The biochemical investigations were carried out with the help, encouragement, and supervision of Dr. C. H. Lack in the Wellcome Research Laboratories, Institute of Orthopaedics. The cost of this work was met in part by a grant from the Joint Clinical Research Committee of the Royal National Orthopaedic Hospital and the Institute of Orthopaedics.

This work forms part of a thesis accepted for the degree of M.S. by the University of London.

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J. clin. Path. (1961), **14**, 309.

A latex fixation test using British latex and bovine gamma globulin

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SYNOPSIS A British polystyrene latex-bovine gamma globulin fixation test which can be used for serum titrations is described. It is technically easier to perform than the latex fixation test of Singer and Plotz (1956) and appears to be equally sensitive. There was agreement with a sensitized sheep cell agglutination test in 264 of 300 cases (88%).

The agglutination of a suspension of polystyrene particles of uniform size mixed with a solution of human gamma globulin in an alkaline buffer by serum from patients with rheumatoid arthritis was first described by Singer and Plotz (1956). Rheins, McCoy, Buehler, and Burrell (1957) showed that gamma globulin from seven animal species, including the cow, could be used in place of human gamma globulin. Burby and Behr (1958) used the American polystyrene latex manufactured by the Dow Chemical Co.[1] to show that human and bovine gamma globulin gave complete agreement in fixation tests on 26 positive and 34 negative sera.

The American latex has only recently become readily available in Britain but a similar, less expensive suspension has been manufactured by Styrene Products Ltd.[2] Burby and Behr (1959) reported that the British product gave the same results as the American in latex-bovine gamma globulin tests if used in a different dilution, but Coke (1959) found they they agglutinated spontaneously. He used this reaction as the basis of a latex agglutination inhibition test. Agglutination was inhibited by the serum of most patients with arthritis not of rheumatoid type, but not by the serum of most patients with rheumatoid arthritis. The test cannot be used for serum titrations because agglutination occurs in the test system in the absence of serum.

By analogy with antigen-antibody reactions it seemed possible that spontaneous agglutination could be inhibited by excess of either latex or bovine gamma globulin. A non-agglutinating mixture could be used for serum titrations if its reactivity were retained.

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[1] Distributed by Difco Laboratories
[2] Distributed by British Drug Houses, Ltd.

PRELIMINARY INVESTIGATIONS

Borate-saline buffer *p*H 8·2 (Singer, 1958) and a stock suspension of British latex (Burby and Behr, 1959) at a final concentration in the test systems of 1 volume in 100 volumes were used in the preliminary investigations (see Appendix). Unless otherwise stated the reactants were mixed in 2½ in. × ¼ in. tubes, heated in a water-bath at 56°C. for two hours, and the presence or absence of agglutination determined with the naked eye after allowing the tubes to stand at room temperature for 18 hours by holding them in a strong oblique light against a dark background.

Mixtures of a constant concentration of latex and a wide range of concentrations of bovine gamma globulin were tested in this way. The results are shown in Table I.

Agglutination did not occur with bovine gamma globulin concentrations above $3{\cdot}1 \times 10^{-1}$ g./100 ml. and below $4{\cdot}9 \times 10^{-5}$ g./100 ml. Agglutination was also absent between concentrations of $6{\cdot}3 \times 10^{-3}$ g./100 ml. and $7{\cdot}8 \times 10^{-4}$ g./100 ml.

Serial dilutions in glycine-saline buffer of sera from patients with rheumatoid arthritis and from normal controls were mixed with equal volumes of various British latex-bovine gamma globulin so that the test systems contained final concentrations of latex and bovine gamma globulin which had been shown not to agglutinate in the absence of serum.

Agglutination failed to occur with any rheumatoid serum tested. At bovine gamma globulin concentrations of $6{\cdot}3 \times 10^{-1}$ g./100 ml. and above. At concentrations between $6{\cdot}3 \times 10^{-3}$ g./100 ml. and $7{\cdot}8 \times 10^{-4}$ g./100 ml. it was possible to obtain titres with a clear end-point. Prozoning did not occur. At concentrations below $4{\cdot}9 \times 10^{-5}$ g./100 ml. a titre

TABLE I

REACTION BETWEEN BOVINE GAMMA GLOBULIN (B.G.G.) AND A CONSTANT CONCENTRATION OF LATEX

B.G.G. Concentration (g./100 ml.)	*Reaction*
2·5	Nil
1·3	,,
$6·3 \times 10^{-1}$	,,
$3·1 \times 10^{-1}$	Agglutination
$1·6 \times 10^{-1}$	,,
$7·8 \times 10^{-2}$	,,
$5·0 \times 10^{-2}$	,,
$3·9 \times 10^{-2}$	,,
$2·5 \times 10^{-2}$	,,
$2·0 \times 10^{-2}$	,,
$1·3 \times 10^{-2}$	,,
$9·8 \times 10^{-3}$	,,
$6·3 \times 10^{-3}$	Nil
$4·9 \times 10^{-3}$	,,
$3·1 \times 10^{-3}$	,,
$2·4 \times 10^{-3}$	,,
$1·6 \times 10^{-3}$	,,
$1·2 \times 10^{-3}$	,,
$7·8 \times 10^{-4}$	,,
$3·9 \times 10^{-4}$	Agglutination
$2·0 \times 10^{-4}$	,,
$9·8 \times 10^{-5}$	,,
$4·9 \times 10^{-5}$	,,
$2·4 \times 10^{-5}$	Nil
$1·2 \times 10^{-5}$	,,
$6·1 \times 10^{-6}$	,,
All lower concentrations tested	,,

could be obtained but there was a prozone with many of the positive sera tested. The prozone became more marked and the titre became lower as the bovine gamma globulin concentration was reduced. A final concentration of bovine gamma globulin in the test system of 5 mg./100 ml. gave the highest titres with the majority of the positive sera tested and was selected for use.

The titres obtained using the technique of Burby and Behr (1958 and 1959), but using a final concentration of bovine gamma globulin of 5 mg./100 ml. instead of 25 mg./100 ml., were compared with the titres obtained after heating the reactants as follows:

1 Unheated latex suspension and bovine gamma globulin solution were mixed and added to unheated positive sera and to sera heated to 56°C. for one hour.

2 Latex suspension and bovine gamma globulin solution were heated separately to 56°C. for one hour, cooled and added to heated and unheated sera.

3 Latex suspension and bovine gamma globulin solution were mixed, heated to 56°C. for one hour and for two hours and added to unheated sera and to sera which had been heated to 56°C. for one hour, both undiluted and in serial dilutions in buffer.

The highest titres were obtained when the latex-bovine gamma globulin mixture was heated to 56°C. for two hours, cooled, and added to the serum dilutions. Heating the sera undiluted or in serial dilutions in buffer did not alter the titres.

On the basis of these results a latex fixation test with British latex and bovine gamma globulin which can be used for serum titrations has been devised. It has been compared with a sensitized sheep cell agglutination test.

METHODS

The latex fixation test is described in full in the Appendix. The sensitized sheep cell agglutination test was that of Greenbury (1957), with the exception that all sera were absorbed for two hours once only with washed unsensitized sheep cells (after Ball, 1950). Occasionally absorption was incomplete. A differential agglutination titre was then calculated (Rose, Ragan, Pearce, and Lipman, 1948). The tests were performed in plastic agglutination trays. Sheep serum was not used.

The two tests were performed on sera referred for Rose-Waaler tests within 48 hours of each other and usually on the same day. The latex fixation tests were read by the author and the sensitized sheep cell agglutination tests were read independently by Dr. J. L. Withey of this department. Neither was aware of the clinical diagnoses at the time of reading.

RESULTS

The titres obtained in the two tests on 300 sera are shown in Table II.

TABLE II

TITRES IN LATEX AGGLUTINATION AND SENSITIZED SHEEP CELL AGGLUTINATION TESTS IN 300 CASES

Latex Titre	*Sensitized Sheep Cell Agglutination Test Titre*								
	<16	*16*	*32*	*64*	*128*	*256*	*512*	*1,024*	*2,048*
5,120	—	—	—	—	—	—	1	1	1
2,560	—	—	—	—	—	1	1	1	2
1,280	—	—	1	1	2	1	—	4	—
640	2	1	3	8	7	1	2	—	1
320	4	1	3	5	4	2	3	2	2
160	5	2	—	1	4	3	2	—	1
80	7	—	—	5	2	1	1	—	1
40	7	1	—	1	1	1	—	—	—
20	7	2	—	—	1	2	—	—	—
10	18	—	—	—	—	—	—	—	—
<10	143	5	4	1	2	1	—	—	—

The reading of the end-point in the latex fixation test showed good reproducibility when repeated 'blind' by one observer. Variation between observers was very rarely more than one dilution. Variation between repeated tests on the same serum was never more than one dilution.

In the sensitized sheep cell agglutination test agglutination at an initial serum dilution of 1 in 32 or greater was considered positive.

Conventionally the latex fixation test is considered positive if agglutination occurs at an initial serum dilution of 1 in 20 or greater. Table III shows the correlation between the tests using these criteria. There is agreement in 84·3% of cases.

TABLE III

COMPARISON OF 300 DUPLICATE TESTS

Latex Test	*Sensitized Sheep Cell Agglutination Test*	
	Positive	*Negative*
Positive[1]	87	39
Negative	8	166

[1]Latex fixation 1 in 20 or greater positive.

It will be seen that the latex test is nearly always positive when the sensitized sheep cell agglutination test is positive and is often positive when the latter is negative. This might be due to greater sensitivity of the latex test or to false positive reactions.

It would be convenient to reduce the sensitivity of the latex test as closely as possible to that of the sensitized sheep cell agglutination test. This can be done by assuming the latex test to be positive at an initial serum dilution of 1 in 80 or greater. Table IV shows the correlation between the tests using these criteria. There is agreement in 264 of 300 tests (88%).

TABLE IV

COMPARISON OF 300 DUPLICATE TESTS

Latex Test	*Sensitized Sheep Cell Agglutination Test*	
	Positive	*Negative*
Positive[1]	82	22
Negative	14	182

[1]Latex fixation 1 in 80 or greater positive.

An attempt has been made to analyse the clinical conditions of the patients whose sera have given different results by the two tests. This has been done by reference to the clinical diagnoses given on the request forms and, where necessary, by discussion with the clinicians.

Of the 14 cases in Table IV positive by the sensitized sheep cell agglutination test but negative by the latex test, 11 were cases of definite or probable rheumatoid arthritis. The remaining three patients had no arthritis but were coalworkers with chest radiographs considered by Dr. A. Caplan to be typical or suggestive of rheumatoid pneumoconiosis (Caplan, 1953). Of the 22 cases positive by the latex test but negative by the sensitized sheep cell agglutination test, 17 were cases of definite or probable rheumatoid arthritis, two were coalworkers without arthritis but with chest radiological appearances suggestive of rheumatoid pneumoconiosis, and three cases remain undiagnosed. Of these, one is thought to have polyarteritis, one is thought to have systemic lupus erythematosus, and the third, a boy of 13, had a transient polyarthritis of undetermined origin.

DISCUSSION

The reactions between various concentrations of bovine gamma globulin and a constant concentration of latex shown in Table I took place at *p*H 8·2, well above the isoelectric point of bovine gamma globulin. It is therefore unlikely that the agglutination which occurred was due to insolubility of the protein. There is good evidence that polystyrene particles are 'coated' by globulins. If bovine gamma globulin molecules and polystyrene particles are polyvalent in their mutual affinity, partially coated particles would be expected to interact and agglutinate. No observable reaction would be expected in the presence of excess of either. In fact, agglutination occurred at many concentrations between $6{\cdot}3 \times 10^{-1}$ g./100 ml. and $2{\cdot}4 \times 10^{-5}$ g./100 ml., and no reaction was observed at higher and lower concentrations. However, it has not been possible to find a simple explanation for the absence of agglutination at bovine gamma globulin concentrations between $6{\cdot}3 \times 10^{-3}$ g./100 ml. and $7{\cdot}8 \times 10^{-4}$ g./100 ml.

Greenbury (1960) has shown that his modification of the Rose-Waaler test (Greenbury, 1957) gives a very high positive rate in rheumatoid arthritis and a low proportion of false positives, and therefore forms a suitable base with which to compare the efficiency of other tests. The titres obtained with Greenbury's modification of the sensitized sheep cell agglutination test in the present series showed no correlation with the titres obtained with the British latex-bovine gamma globulin test. A lack of correlation of titres has been reported by others using American latex and human gamma globulin as the reagents, for example, Steinberg, Roberts, and Lock (1959). Greenbury compared his test with the original latex fixation test of Singer and Plotz (1956). Sera

were tested at a single dilution of 1 in 20 only. He found agreement in 2,893 of 3,222 tests (89·8%). There was agreement in 264 of 300 tests (88%) in the present series, suggesting that the British latex-bovine gamma globulin test is approximately as sensitive as the American latex-human gamma globulin test.

The advantages of the technique presented in the Appendix are that, instead of incubating all the tubes containing serum dilutions at 56°C. for two hours as in the original method of Singer and Plotz (1956), it is necessary to incubate only a mixture of latex and bovine gamma globulin in bulk, and centrifuging is unnecessary. The reagents are easily obtainable and cost less than 3s. for the titration of 100 sera. Prozoning has not occurred with any of the sera tested.

I wish to thank Dr. J. L. Withey for reading the sheep cell agglutination tests, for permitting me to use sera referred to him, and for his interest and helpful advice.

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ADDENDUM

Since this paper was submitted for publication the test described has been compared with the RA-test (Hyland) which uses human gamma globulin as the reactant. Of sera from 141 subjects, 53 were positive and 82 negative by both tests. Two sera were positive at a titre of 1 in 80 in the latex-B.G.G. test and four sera were weakly reactive in the RA-test but negative in the other test. There was agreement in 135 of the 141 cases (95·7%).

APPENDIX

REAGENTS

GLYCINE-SALINE BUFFER *p*H 8·2 An 0·1 M glycine solution is adjusted to *p*H 8·2 by the addition of N sodium hydroxide, and 10 g. of sodium chloride added to each litre.

The buffer may be stored at 2 to 4°C. for several months, but must be discarded if turbidity develops.

STOCK GAMMA GLOBULIN SOLUTION An 0·1% solution of Armour bovine gamma globulin fraction II in buffer.

The gamma globulin solution may be stored at −20°C. for several months, preferably in separate small quantities to avoid repeated thawing and freezing.

STOCK LATEX One volume of B.D.H. polystyrene latex (30% suspension) is added to 50 volumes of distilled water and filtered through a Whatman No. 40 filter paper. The density is adjusted by the addition of distilled water so that when 1 volume of stock latex is added to 10 volumes of buffer the dilute latex gives a light transmission of 33 to 35% in a 1 cm. cell at 650 μ.

The stock latex may be stored at 2 to 4°C. for several months but must be discarded if clumps develop.

METHOD

A latex-globulin mixture is made by mixing 2 volumes of stock latex with 93 volumes of buffer and adding 5 volumes of stock gamma globulin solution. Ten millilitres of this mixture is required for each serum. Sufficient mixture for the sera to be tested is placed in a suitable stoppered container, heated in a water-bath at 56°C. for two hours and allowed to cool.

The sera are diluted serially in buffer from 1 in 40 to 1 in 20,480 in clean $\frac{3}{4}$ in. × $2\frac{1}{2}$ in. tubes and 1 ml. is left in each tube. Control tubes containing 1 ml. of buffer only are also prepared.

One millilitre of heated and cooled latex-globulin mixture is added to each tube.

Readings are made after the tubes have stood at room temperature for 18 hours.

Agglutination of minor degree is most easily seen by holding the tube in a strong oblique light and inspecting it against a dark background. A hand lens is not used. Agglutination is present if there is complete sedimentation of the latex particles or if discrete particles can be seen in suspension. Agglutination is absent if the suspension remains uniformly milky, even if a small amount of sediment is present.

The titre of a serum is the greatest initial serum dilution showing agglutination. Agglutination at a dilution of 1 in 80 or greater is considered a positive test. The control tubes should show no agglutination.

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The testing of sterilizers

2 Thermophilic spore papers[1]

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SYNOPSIS Satisfactory spore preparations for testing hospital sterilizers can be made from *B. stearothermophilus* and comparatively simple methods for loading and drying the papers are considered to be valid. Each batch of spore papers should be calibrated by plotting dose-response curves for exposure to steam. A minimum standard in terms of the L.D.50 to moist heat is proposed and confidence limits suggested when such papers are used in pairs or small multiples.

In a previous communication (Kelsey, 1958) attention was drawn to the dangers of testing the efficiency of steam sterilizers by using bacterial preparations of inadequate resistance. It was suggested that when cultural tests are required only preparations of known heat resistance should be used. The present paper describes how such preparations may be made and standardized.

PREPARATION OF SPORE PAPERS

It was decided to use *Bacillus stearothermophilus*, an aerobic spore-bearing thermophile which is well known in the canning industry as the cause of 'flat sours'. Several standard freeze-dried strains are available and the literature suggested that it would have a suitable resistance to heat.

Many techniques were tried in an attempt to produce a good yield of spores. Even using artificial aeration, liquid culture media did not produce such good yields as solid media and eventually the medium chosen was that of Williams, Franklin, Chapman, and Clegg (1957) and called by them 'B.S. medium'. This medium and the details of its use to produce a spore suspension are described below. The technique differs in some respects from that suggested by the Nuffield Provincial Hospitals Trust (1958) and by Farr (1959) but the results have been consistently satisfactory.

The application of 'heat shock' by heating the suspension to 100°C. for five minutes is important. This process applied immediately after harvesting destroys the vegetative bacteria and so enables the viable count to be a true count of spores alone (Curran and Evans, 1947).

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Although the same workers (Evans and Curran, 1960) consider that such spore suspensions once 'heat shocked' retain their viability after prolonged storage at low temperatures (provided the *p*H is around neutrality), 'heat shock' has always been reapplied just before the impregnation of spore papers, if this is not done at once. This procedure kills any extraneous vegetative organisms that may have contaminated the suspension during storage. It also means that the pre-impregnation viable count of spores will be maximal.

STRAINS The following strains of *Bacillus stearothermophilus* were found satisfactory: N.C.I.B. 8919, N.C.T.C. 10003, N.I.R.D. 202.

INOCULUM Initially a suspension containing spores was made by growing the organism on nutrient agar at 56°C. for five days. In all later productions an existing spore paper was used. The primary inoculum is heated to 121°C. for five minutes, and incubated overnight in broth at 56°C. This culture is then used to inoculate several tubes of broth which are incubated at 56°C. for six hours, and after concentration by centrifugation a heavy suspension is obtained from which the main cultures in Roux bottles are inoculated. It is more satisfactory always to go back to an early batch of spore papers for the inoculum, having found that this gives a good yield, rather than to use the last paper of the previous batch to start the next one.

MEDIUM After much experimentation it was found that good results were consistently obtained by using the 'B.S. medium' of Williams *et al.* (1957) which has the following composition: Oxoid tryptone, 3 g., oxide peptone, 6 g., Yeastrel, 3 g., Lab-Lemco, 1·5 g., New Zealand agar, 25 g., aqueous solution containing 0·001 g. per ml. Mn^{++}, 1 ml., distilled water, 1,000 ml. The final *p*H is adjusted to 7·0. This medium was used to make Roux bottles.

INCUBATION Bottles are incubated for five to seven days at 56°C. The bottles are incubated with the agar downwards and sloped so that excess moisture collects at one end. If necessary, a little sterile distilled water is added from time to time to prevent the drying of the agar. It is important to maintain aerobic conditions by using a loose cottonwool plug or metal cap to close the Roux bottles.

HARVESTING If a stained film shows good sporulation (over 80%) the growth is harvested by shaking with sterile distilled water and sterile glass beads. The crude suspension is heated at 100°C. for five minutes, shaken with beads on a mechanical shaker to break up clumps, and filtered through glass wool. It is washed three times and resuspended in sterile distilled water. A wet preparation is then examined to check that the suspension is smooth and free from clumps. If this is satisfactory the suspension is stored at + 4°C. until required. If long storage is expected a drop of a mixture of equal volumes of toluene and carbon tetrachloride is added, or the spores can be suspended in methanol.

PREPARATION OF PAPERS The suspension is heated at 100°C. for five minutes, counted by the method of Miles and Misra and diluted until the required number of spores is contained in 0·033 ml. The suspension is faintly tinted with the edible dye 'apple green' and used to impregnate Whatman's A.A. discs, 6 mm. in diameter. These discs absorb 0·033 ml. of water.

The discs are placed in a petri dish in shallow layers and flooded with the suspension (the dye prevents any disc being missed). Excess liquid is drained off and the disc dried at room temperature and stored at the same temperature until required.

LOADING THE SPORE PAPERS At first strips of filter paper were used to carry the spores, but cutting them proved very laborious and it was more convenient to use ready prepared discs made of thick filter paper. Such discs are available as Whatman's antibiotic assay (A.A.) discs. They are very uniform and careful experiment showed that the 6 mm. discs regularly absorb 0·033 ml. of water from their air-dried state. This greatly facilitates loading them with spores. The discs can be sterilized by hot air or ethylene oxide, but experience has shown this to be unnecessary. Many hundreds of discs which had not been sterilized before impregnation failed to yield any contaminants more resistant than the *B. stearothermophilus* spores used.

If only a few discs are to be made each can be loaded by dropping from a calibrated dropper or by individual dipping in a suspension. With large batches this is too laborious and a technique in which discs in bulk are flooded with suspension is to be preferred. Such a technique, is, however, subject to criticism on at least two grounds. The disc may not take up the suspension uniformly, those moistened first may abstract spores from suspension soaking through them, so that discs moistened later may contain fewer spores. In addition, when such bulked spores are dried those on the outside of the heap will dry last, moisture will soak through them from those below and again the spore distribution may be affected. Thus the reliability of the method of bulk loading needed to be investigated.

It was originally hoped that spores could be recovered quantitatively from impregnated spore papers, so that the efficiency of various methods of loading could be directly estimated. Despite repeated attempts no such quantitative recovery technique could be devised. This was because spores were caught up in the fibres of the filter paper. This could be seen by microscopic examination of the pulp resulting from shaking, boiling, blending, or other disintegrative procedures. Another approach had to be adopted. A spore suspension was counted and a dilution made so that on average 0·5 to 0·75 spores should be present in 0·033 ml. Thus if a group of discs were impregnated with such a suspension and subsequently cultured most, but not all, should yield growth. If the loading method was not uniform the proportion of positive discs should fall, since some would have several spores and some none.

In a typical experiment a row of 50 A.A. discs was arranged in 'rouleaux' formation in a petri dish. A spore suspension was prepared as described above and tinted green with the edible dye 'apple green' which had previously been shown not to be toxic. One end of the row of discs was flooded with suspension and more was added until the dye had spread to the far end. The first, middle, and last 10 discs were cultured in nutrient broth for five days at 56°C.

TABLE I

		Number Yielding Growth
First 10		8
Middle 10		8
Last 10		7
Mean 7·7	$\chi^2 = 0{\cdot}88$	$P = 0{\cdot}6$

This difference is not significant.

In another typical experiment 20 A.A. discs were individually dipped in the suspension and 20 were impregnated in bulk by adding suspension to them in a universal container until the dye appeared to have reached all the discs and a little surplus fluid was visible. Each group was cultured as before.

TABLE II

		Number Yielding Growth
Individual dipping		18
Bulk flooding		14
Mean = 16	$\chi^2 = 1$	$P = 0{\cdot}3$

This difference is not significant.

It was concluded from these and similar experiments that the bulk impregnation of spore papers is a valid procedure.

DRYING THE SPORE PAPERS Apart from the criticism mentioned previously, that discs flooded and dried in bulk might have their spore loads altered in the drying process, it is possible that while the impregnated papers are being dried a proportion of the spores will be killed

so that the final spore population may not be that intended. Two experiments were made to test these points. In the first, 40 A.A. discs were impregnated in bulk using the same suspension as previously and dried overnight at room temperature. Twenty were arranged in a heap; 20 were arranged individually in rows. All were cultured for five days at 56°C.

TABLE III

	Number Yielding Growth
Heap	20
Rows	14
Mean = 17 $\chi^2 = 1{\cdot}09$	$P = 0{\cdot}3$

This difference is not significant.

In the second experiment a batch of discs was impregnated in bulk and groups of 20 were cultured wet and after drying overnight at various temperatures.

TABLE IV

	Number Yielding Growth
Cultured wet	18
Dried at room temperature	16
Dried at 37°C.	13
Dried at 56°C.	14
Mean = 15 $\chi^2 = 1$	$P = 0{\cdot}8$

This difference is not significant.

Thus the use of dried discs would seem to be justified.

STORAGE OF SPORE PAPERS Spore papers made in November 1957 were still viable in June 1960; it was not possible to compare precisely their heat resistance at the beginning and end of this period, since the need for relatively precise standardization methods was not appreciated until late in their history, and at first an 'all-or-none' method of standardization was used. It is probable that on this standard there is a slow fall in resistance, but, as discussed below only further work using more refined techniques will show whether this is true.

RECOVERY OF SPORE PAPERS In practice spore papers are inserted into test loads and cultured after exposure to a sterilization procedure. It was thought possible that the method of culture was important. Experiments were therefore made to test various media, temperatures of incubation, and conditions of culture, using a group of discs impregnated with a nominal load of 100,000 spores and heated for a time calculated to yield a proportion of positive cultures just below 100%. A representative set of results is given below.

TABLE V

Results (Groups of 20 Discs)	*Number Yielding Growth*
Nutrient broth ½ in. depth 50°C.	20
Nutrient broth ½ in. depth 56°C.	20
Nutrient broth ½ in. depth 60°C.	20
Nutrient broth 3 in. depth 56°C.	19
Oxoid dextrose tryptone broth ½ in. depth 56°C.	20
Nutrient broth 56°C. immediately	20
Nutrient broth 56°C. after 1 week	17
Nutrient broth 56°C. after 2 weeks	20
Nutrient broth 56°C. after 3 weeks	18
Nutrient broth 56°C. after 4 weeks	19

It will be seen that the cultural conditions used did not affect the results and that there is no evidence that discs should be cultured within a day or so of exposure.

In all experiments made with spore papers incubation was continued for five days, although it was only very rarely that new positive cultures appeared later than the third day. It is possible that had incubation been extended an occasional new positive would have appeared, but five days was chosen as a reasonable time in practice. It was thought that it was more important to calibrate the overall method of using cultural tests than to try to establish the limits of late recovery, which could easily become quite unrealistic.

STANDARDIZATION OF SPORE PAPERS

Since the object of using thermophilic spore preparations was to ensure adequate heat resistance, it was necessary to lay down some standard for such preparations. This problem became the more pressing when spore papers were commercially produced.

The only precedent available was the standardization of spore suspensions for use in the canning industry. Many elaborate and precise methods are available and a suspension can be characterized by its survival curve, by its thermal resistance curve, or by various mathematical indices (*e.g.*, Schmidt, 1957). All of these suffer from the same disadvantage: they apply to a smooth suspension rather than to the impregnated spore paper. If it is assumed that the resistances of individual spores are normally distributed, the overall resistance of a very large sample should be relatively uniform, since at least one very resistant spore should be in the total spore population comprising the sample. In terms of the making of spore papers this would mean smooth suspensions and the uniform loading of papers. In fact suspensions are seldom smooth and in the previous section it was seen that the processes of loading and drying the paper discs themselves are possible sources or irregularity.

Had it been possible to recover spores quantitatively from the spore papers after impregnation, survival curves, relating the numbers of surviving spores to the time of exposure at a given temperature, could have been plotted and standardization could have been defined in terms of the decimal reduction time.

Because of these difficulties it was decided to treat each spore paper as a biological unit and to attempt to plot dose-response curves against exposure to heat. From this a total picture of the behaviour of the batch of spore papers could be obtained.

It was thought that from this information an L.D. 50 could be deduced which could provide a standard for heat resistance.

In these experiments groups of spore papers, individually wrapped in paper envelopes, were exposed to steam at 121°C. for various times in a high-speed instrument sterilizer, which conformed in all significant details to British Standard Specification 3233:1960.

GENERAL TRIAL OF THE METHOD Determinations were made on the same batch of spore papers on three separate occasions, with the following results:

TABLE VI

	5 May 60		11 May 60		12 June 60	
Number in Group	*20*	*%*	*10*	*%*	*20*	*%*
Survivors 2 min.	20	100	10	100	20	100
Survivors 4 min	16	80	9	90	20	100
Survivors 6 min.	7	35	4	40	7	35
Survivors 8 min.	0	0	0	0	0	0

Dose-response curves plotted from these results are shown in Figure 1. There is a close correspondence between the curves. The L.D. 50 is virtually the same for each, namely, about 5½ minutes.

COMPARISON OF SEVERAL BATCHES OF SPORE PAPERS Since it seemed that the dose-response curve was likely to give useful results, three commercial suppliers of spore papers were asked for 100 papers each. Dose-response curves at 121°C. were then plotted, using groups of 20 papers at each exposure, for the three commercial batches, P, Q, and R, a current batch 4, a very recently prepared batch 5, and the oldest surviving batch A3, which was made in November 1957 and was 30 months old at the time of retesting. The results are given in the tables below.

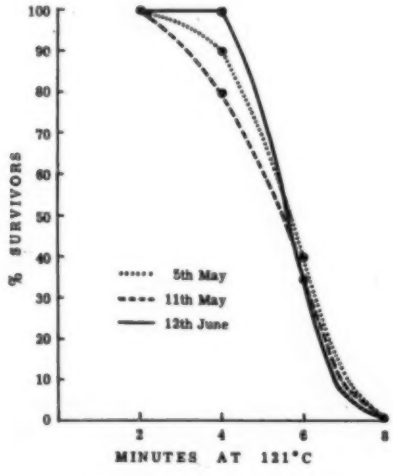

FIG. 1. *Dose-response curve of batch 4 spore papers determined on three separate occasions.*

TABLE VII

	Strain	*Date*	*Origin*
A3	N.C.T.C.9996	Nov. 57	J.C.K.
4	N.C.I.B.8919	Nov. 59	J.C.K.
5	N.I.R.D.202	July 60	J.C.K.
P	N.C.I.B.8919	May 60	Commercial
Q	N.C.I.B.8919	April 60	Commercial
R	N.C.T.C.10003	May 60	Commercial

TABLE VIII

Survivors out of 20

Min.	*A3*	*4*	*5*	*P*	*Q*	*R*
2	16 (80)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)
4	11 (55)	20 (100)	19 (95)	9 (45)	20 (100)	13 (65)
6	4 (20)	7 (35)	10 (50)	1 (5)	19 (95)	0 (0)
8	0 (0)	0 (0)	4 (20)	0 (0)	6 (30)	0 (0)
10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Percentages are in brackets

It will be seen that tolerably smooth curves are given by each batch, considering the necessarily rather crude technique used. The top of the curve for A3 is incomplete; exposures of less than two minutes were not considered reliable, and were not attempted.

The curves for batches P, Q, and 4 were roughly parallel. Each of these batches was made from the same strain of *B. stearothermophilus*, N.C.I.B. 8919. Batch R, made from N.C.T.C. 10003, and Batch 5, made from N.I.R.D. 202, do not show a very different slope, but Batch A3 made from N.C.T.C. 9996 shows a much more gentle slope than the others.

Two possible explanations for these differences could be given. The curve might be characteristic of the strain used, or of the age of the spore papers, A3 being two years older than the other batches. There is not enough evidence to settle this point finally, but the first hypothesis seems preferable. First N.C.T.C. 9996 was discarded very early in the spore making programme just because it was found to give very variable results. Secondly, although standardization of papers was not attempted until 1960, there is a record of survival experiments made when the papers were new. These were crudely performed, only six papers being used per group and the heating was done in an unmodified pressure cooker. At 121°C. six out of six papers survived five minutes and three out of six survived 10 minutes.

When these are plotted, to give the continuous line on Fig. 2, the slope does not differ widely from that obtained two years later. Only repeated detailed calibration of large batches of papers over several years will settle this point.

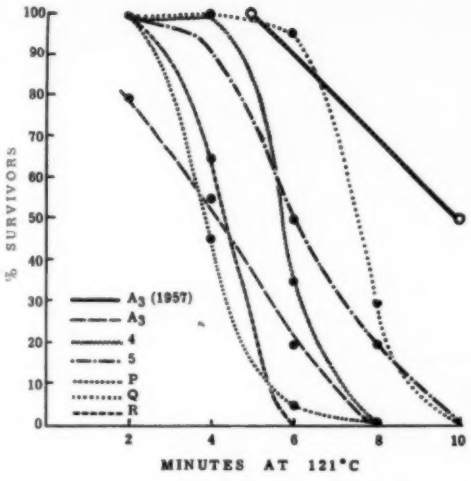

FIG. 2. *Dose-response curves of different batches of spore papers: tested June and July 1960. (Also plotted is the rough curve obtained from two isolated observations on batch A3 in December 1957.)*

USE OF THE L.D.50 TO DEFINE A STANDARD At present it seems reasonable to assume that the slope of the dose-response curve is characteristic of the strain and remains reasonably constant at least for a period of months. If this is accepted the L.D.50 could be used to characterize a batch of spore papers and a minimum L.D.50 could be required from a batch made from that strain. The problem is then to choose such a minimum standard. Before this can be attempted the degree of safety expected from a particular value of L.D.50 must be considered.

In Fig. 1 the L.D.50 for the curve of 11 May 1960 is five and a half minutes. At four minutes 90% survival was obtained and at seven minutes 10%. It could thus be claimed that if a single spore paper were to be used in a test sterilization at 121°C. and if it was in fact sterilized, there is a probability of 0·9 that the load had received at least four minutes' exposure at 121°C. Expressed differently, one such test in 10 might give a falsely reassuring result if four minutes is taken as an adequate time. If a pair of papers were to be used and both were in fact sterilized, a false result could be given once in 100 tests. For three papers the chance would be once in 1,000 tests and so on.

From the same curve it might be deduced that at the three minutes' level the probability is 0·98, but the accuracy of deductions from such sigmoid curves is least near the ends and it is probably unwise to go above 90% or below 10%.

POSSIBLE USE OF PROBABILITY CURVES More refined statistical methods would allow more accurate probabilities to be deduced.

The analysis by the method of Schmidt (1957) following Reed (1936) of the pooled results on Batch 4 spore papers is as follows:

	5 May	*11 May*	*12 June*	*Total*	
Number in Group	*20*	*10*	*20*	*50*	*%*
Survivors 2 min.	20	10	20	50	100
Survivors 4 min.	16	9	20	45	90
Survivors 6 min.	7	4	7	18	36
Survivors 8 min.	0	0	0	0	0

Time	+	−	*m*	*n*	*p*
2	50	0	113	0	0·9
4	45	5	63	5	8·6
6	18	32	18	37	67
8	0	50	0	87	99

where + = number growing
− = number sterile
m = running total of + from bottom
n = running total of − from top

and p = probability of sterility $= \frac{n+1}{m+n+2}$

These are shown plotted on probability paper in Fig. 3. The points are not linear but the best straight line has been drawn. It will be seen that the L.D.50 is as before, five and a half minutes.

The probability of survival at four minutes is 0·93 and at three minutes 0·98, and at two and a half minutes 0·99. From this it could be stated that if

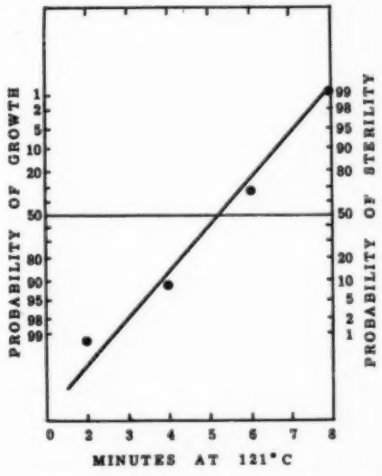

FIG. 3. *Probability of sterility or growth plotted against time of exposure at 121°C. Pooled results on batch 4.*

such a paper was sterilized, only on one occasion in 100 would it have received less than two and a half minutes. For a pair of papers it would be once in 10,000 times and so on.

The plot of Fig. 3 is from pooled data, and represents groups of 50 papers heated for each time interval. Similar plots from the commercial batches, limited to 20 papers per group, were too irregular to use. The more refined analysis is sensitive to smaller variations and needs larger groups of test object than are likely to be available in practice: it also involves much labour. It is probably better to continue to use the cruder but more convenient dose-response curve to define a batch of spore papers.

A SIMPLE STANDARD PROPOSED From the data presented in the earlier paper (Kelsey, 1958), three minutes at 121°C. might be considered as a bottom limit for a test preparation. It would be preferable to take five minutes as giving a wider safety margin. Figure 4 shows the pooled dose-response curve of the three estimations of Batch 4. This gives a 0·9 probability at four minutes. Parallel to this curve are drawn hypothetical curves (W and X) to give the same 0·9 probability at three minutes and five minutes. From these L.D.50s of four and a half and six and a half minutes are deduced, which will be the minimum to give a 0·9 probability of surviving three and five minutes, respectively.

Thus, for this strain a satisfactory batch of spore papers should have a L.D.50 of at least six and a half minutes at 121°C. (or four and a half minutes if the less strict standard is accepted). Such a batch, if used in pairs, will give a potentially misleading result once in 100 times.

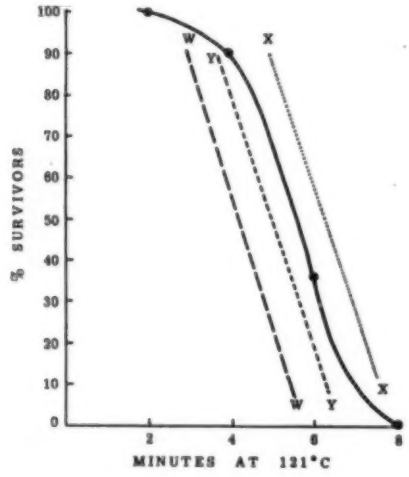

FIG. 4. *Dose-response curve from pooled results on batch 4 with hypothetical curves to show three possible standards.*

In view of the general crudity of the methods used to prepare and test spore papers two further simplifications can probably be accepted. First, a round figure of five minutes for the minimum L.D.50 could be used. Secondly, the standard could be defined more simply by requiring that after being heated for five minutes at 121°C. not less than 50% of a group of, say, 25 papers, should survive, always assuming that the dose-response curve did not depart significantly from that of the strain just considered, namely, N.C.I.B. 8919. Such papers used in pairs would give an assurance of an exposure to 121°C. for a minimum of about three and a half minutes with a 1 in 100 chance of being misleading. This can be deduced from the third hypothetical curve Y. Since a sterilizer is unlikely to be judged on a single test, serious error is unlikely to occur.

INFLUENCE OF THE SPORE LOAD PER PAPER UPON L.D.50 If this crude but simple standard of a minimum survival of 50% after five minutes at 121°C. is accepted, makers of spore papers will need to consider how this standard can be reached. Intuitively it seemed that one way to alter this resistance would be to alter the number of spores per paper, which up to now had been arbitrarily standardized at 100,000.

A further experiment was made in which a single spore suspension was used to load papers with a nominal 1,000, 10,000, and 1,000,000 spores respectively. The resulting papers were then heated at 121°C. in steam for various times.

	1,000		*100,000*		*1,000,000*	
Number in Group	*20*	*%*	*20*	*%*	*20*	*%*
Survivors 2 min.	12	60	20	100	20	100
Survivors 4 min.	4	20	18	90	20	100
Survivors 6 min.	2	10	8	40	18	90
Survivors 8 min.	0	0	0	0	7	35
Survivors 10 min.	0	0	0	0	0	0

These results are plotted in Fig. 5.

The dose-response curves are approximately parallel. At 1,000 and 1,000,000 spores per paper the L.D.50 is two and a half and seven and a half minutes respectively.

Thus in practical circumstances once a suspension has been made, some of it could be used to make trial spore papers loaded with several different numbers of spores. Batches of these could be tested for survival of five minutes at 121°C. and from the results of this relatively simple experiment a loading

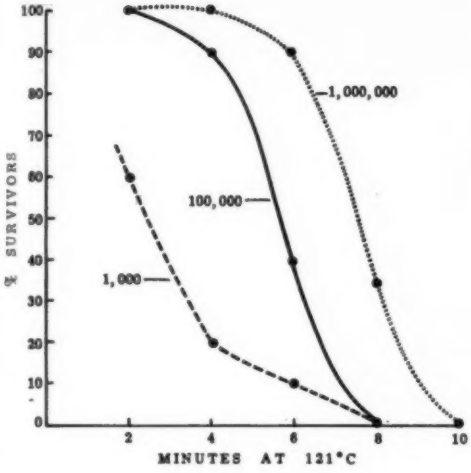

FIG. 5. *Dose-response curves for different levels of loading papers with spores.*

could be selected which would give the production batch the required minimum survival.

It is suggested that if strains of *B. stearothermophilus* are used whose behaviour does not differ significantly from that of N.C.I.B. 8919, a recently made batch of spore papers having a survival of not less than 50% after exposure to steam at 121°C. for five minutes will give acceptable results, if used in pairs and if the possibility of error once in 100 tests is recognized. Until the ageing of spore papers has been studied in greater detail it is probably advisable to recalibrate each batch every six months.

All experience gained since the publication of the earlier paper has confirmed two of the opinions therein expressed. First, for routine sterilizer testing chemical indicators are the most generally useful: bacteriological tests should be kept for special purposes and should be made by experienced people. Secondly, where spore preparations are used these must be carefully standardized, and ideally should be provided by a reference laboratory or from commercial sources.

I am indebted to Professor Robert Knox for advice and helpful criticism, to Mr. Alan Saied for technical assistance, and to Messrs. Allen and Hanbury, Messrs. Oxoid, and the Director of the Southern Group Laboratories for supplies of spore papers.

ADDENDUM

Since the above paper was completed a further calibration of spore papers has been made, five to six months after the first. No further samples of P were available.

Batch No.	*Interval (mth.)*	*Old L.D.50 (min.)*	*New L.D. 50 (min.)*
A3	6	4½	6
4	5	5½	5½
5	5	6	7
Q	5	6	7
R	5	4½	4½

The slopes of the new calibration curves did not differ markedly from those previously obtained.

These results suggest that spore papers once found satisfactory have a shelf life of at least six months. Further estimations on these papers will be made from time to time.

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Technical methods

An apparatus for measuring the tensile strength of blood clots

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SYNOPSIS An apparatus is described which uses the principle of the ballistic pendulum to measure the tensile strength of blood clots formed in special cuvettes. The method appears to have a reproducibility which would allow a study to be made of the factors influencing clot strength. A brief survey of the findings with normal blood and with blood from cases of thrombosis and haemophilia shows significant differences between the mean values for each group.

The function of blood clots in haemostasis and repair must depend to some extent on their adhesiveness, tensile strength, and elasticity, and it would be of interest to know if variations in these properties are related to abnormal bleeding, thrombosis or delayed healing. Elasticity has been studied by Hartert (1948) and others, using the thromboelastograph, and by Scott Blair and Burnett (1960). Lalich and Copley (1942) estimated 'clot firmness' by measuring the pressure required to force clots through a constricted tube. Morrison and Doppelt (1954) have measured the adhesion of clots to different surfaces.

The measurement of the tensile strength of a whole blood clot is complicated by the fact that it is so friable that the mechanical anchorage to which traction is applied is likely to cause tearing at the site of attachment. In consequence, most previous work has been limited to plasma or fibrinogen clots, which are much tougher than those formed by whole blood. These have been studied by Tarlov, Goldfarb, and Benjamin (1942) who used rubber-covered clamps to grip the clot, and attached weights to its free end, and by Neurath, Dees, and Fox (1943) who incorporated gauze in the clot during its formation and used these points of attachment for suspension and loading.

The apparatus described here was designed to measure the tensile strength of whole blood clots without any previous manipulations. It is applicable to any other coagulable fluid, and can be modified to measure adhesion to a given surface. In measuring tensile strength, however, adhesion of the clot to its container is not a requirement. A brief description of the apparatus is contained in the proceedings of a symposium on the flow properties of blood (Macfarlane, Tomlinson, and Excell, 1960).

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PRINCIPLE

Blood taken by venepuncture is allowed to clot in a special cuvette which has a dumb-bell shaped cavity. The complete cuvette is assembled from two identical halves, the opposing faces of which are flat and fit accurately, the junction between them passing through the mid-point of the narrow, cylindrical 'neck' which connects the two wide cavities of the dumb-bell (Fig. 1). When clotting has occurred, the two half cuvettes are mechanically united by the clot, since its two expanded portions cannot pass through the narrow neck, and traction applied to one half cuvette will be transmitted to the second by the 'isthmus' of clot until it breaks.

The principle of the ballistic pendulum is used to measure the force applied to the clot. One half cuvette is located in a carrier at the end of a light but rigid pendulum (the measuring pendulum) hanging vertically from a bearing. The other half cuvette is located in a similar carrier, also attached to a pendulum arm (the traction pendulum) which swings from the same bearing as the first but is free to move independently of it. The two carriers are adjusted to be in register, so that when the cuvette is put into them the two half cuvettes remain aligned and in contact. The two pendulums are now connected only by the clot which connects the two half cuvettes. An electric winch moves the traction pendulum round the arc of a circle, and the measuring pendulum follows it, drawn by the tension in the clot. The fact that both pendulums have a common axis ensures that the two half cuvettes maintain their alignment until separation occurs. The force necessary to maintain the measuring pendulum at an angle, θ, to the vertical is directly proportional to sine θ, and since this force is transmitted by the clot, the tension in the clot increases as the movement progresses. When the tension is great enough the clot breaks, and the maximum deflection reached by the measuring pendulum is a measure of the maximum tension achieved. This deflection is recorded by the simple method of arranging for a sprag attached to the measuring pendulum to trail over a surface below it, offering little resistance to the forward movement of the pendulum, but preventing any reverse movement. Thus, as soon as the clot breaks, the measuring pendulum becomes stationary at maximum deflection, its position being indicated on a scale.

Since the friction of the bearings and sprag is negligible, the instrument can be calibrated from a knowledge of the dimensions and weight of the measuring pendulum.

CONSTRUCTION

The cuvettes are machined from perspex rod to the sizes indicated in Fig. 1. Perspex coverslips are also provided. The carriers of the pendulum are also machined from perspex, with semicircular grooves to accommodate the flanges of the cuvette. When in the starting position

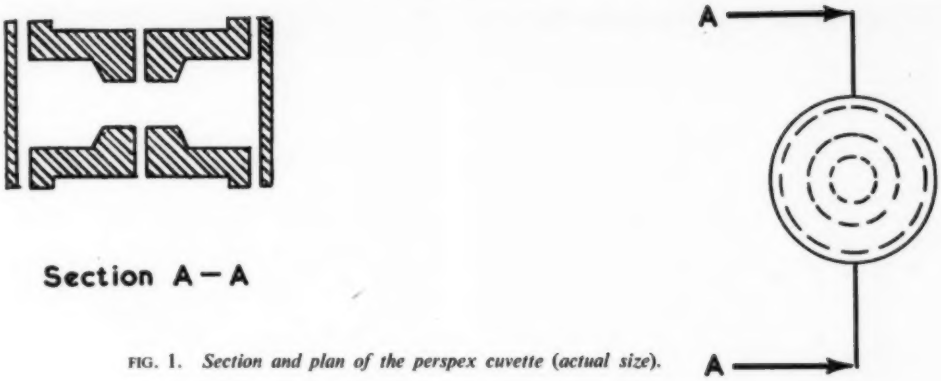

FIG. 1. *Section and plan of the perspex cuvette (actual size).*

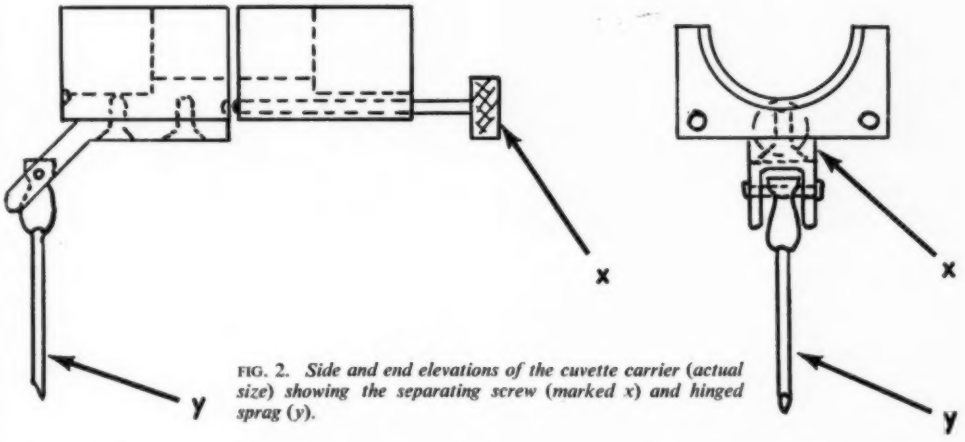

FIG. 2. *Side and end elevations of the cuvette carrier (actual size) showing the separating screw (marked x) and hinged sprag (y).*

the two carriers are so aligned that they form a trough-shaped receptacle into which the cuvette fits accurately. To provide the small, carefully controlled separation of the half cuvettes necessary to break the vaseline seal (see below) a screw in the carrier of the traction pendulum bears on the carrier of the measuring pendulum (Fig. 2).

The traction pendulum is built of brass strip, since its weight is not a consideration, but the measuring pendulum has to be as light as possible, in order to ensure reasonable sensitivity, and sufficiently rigid to prevent lateral displacement. This pendulum consists of a fibre-glass tube, 4 cm. in diameter, with walls 1 mm. thick, and complete with carrier and a half cuvette filled with blood clot, weighs 200 g. Both pendulums are 1 metre long. The traction pendulum is suspended from plain bearings and the measuring pendulum from a needle and cup bearing arranged concentrically, both being supported on a braced upright constructed of Dexion angle strip (see Fig. 3), mounted on a base board, which can be levelled by means of screw legs. Two adjustable stops prevent the measuring pendulum from moving left-handed away from its position of equilibrium. A pointer on the measuring pendulum travels over a scale directly calibrated in grams of tension. Behind the scale is a strip of metal, following the curvature of the arc, and covered with 'elastoplast', forming the surface over which the sprag trails, and into which it digs at the first reversal of movement. The sprag itself is a small steel needle, hinged to the lower end of the measuring pendulum. The sprag can be 'tripped' by the finger to re-set the pendulum to zero after a reading has been taken (Fig. 4).

The cuvette is moved at a constant speed of about 1·5 cm./sec. by an electric winch which can be reversed in order to return the pendulum to its starting point.

OPERATION

The cuvette is carefully washed and dried. After very lightly greasing the faces with vaseline, the half cuvettes are assembled, care being taken to prevent any grease exuding into the bore when the two halves are pressed together. A coverslip is applied, after greasing, to the

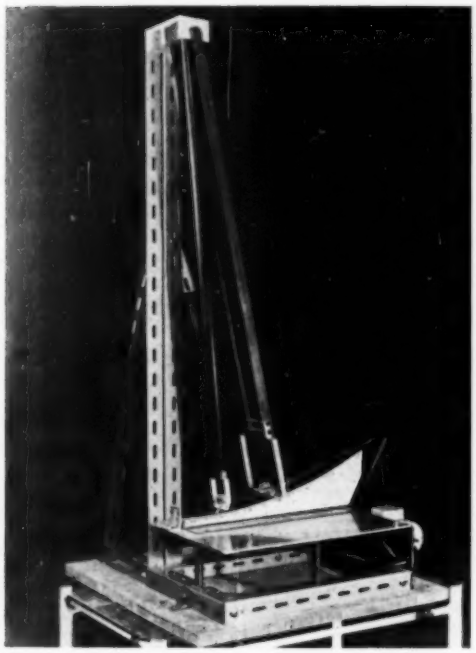

FIG. 3. *The complete tensiometer showing the two pendulums with half-cuvettes in position on the carriers. In this illustration the clot has broken so that the measuring pendulum has come to rest and the traction pendulum has moved away from it.*

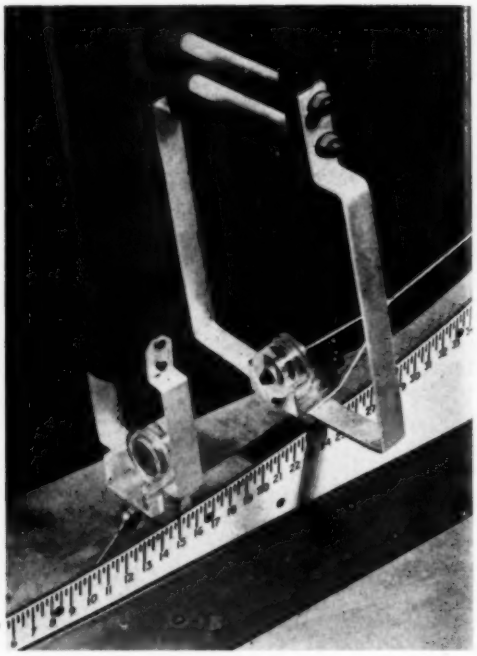

FIG. 4. *Close-up view showing the lower ends of the two pendulums with half-cuvettes located in their carriers. The pointer attached to the measuring pendulum has indicated on the scale the tension in grams at which breakage of the clot had occurred.*

lower end and the assembled cuvette is mounted in a holder which maintains the halves in alignment, and allows filling through the open top. Blood is drawn by syringe, and the cuvette is filled by inserting the needle to the bottom, so that air locks and bubbles are avoided, and expelling blood until it is level with the top; the volume of blood required is about 2·5 ml. The filled cuvette is then incubated at the required temperature, the onset of coagulation noted by tilting, and at the required time after clotting, testing is carried out. A cuvette is placed horizontally in the pendulum carriers shown in figure 2. The vaseline seal is then broken by carefully turning the screw, x, until the half cuvettes are separated by about 0·5 mm., no significant stress being applied to the clot by this movement. Once the seal is broken, the isthmus of the clot is the only physical connexion between one carrier and the other, and traction is applied by switching on the electric winch. At first the two pendulums move together; the clot begins to extend, it breaks, and the measuring pendulum remains at its highest point. The scale reading is recorded, the cuvette halves are removed, the measuring pendulum is returned to zero by releasing the sprag, the traction pendulum by reversing the motor, and the operation can be repeated with the next cuvette. The complete measurement takes about 30 seconds.

RESULTS

Only a few preliminary results will be quoted to illustrate the reproducibility of the method, and the range observed in a small series of normal and abnormal subjects.

NORMAL WHOLE BLOOD The cuvettes were filled directly from the syringe into which the blood was collected, and then incubated. It was usual to make the estimation at one hour after clotting, but, in normal cases, longer incubation periods up to four hours had no apparent effect on the readings.

Reproducibility A series of 12 cuvettes was filled from the same sample of fresh blood and tested after one hour's incubation. The readings ranged from 5·3 to 6·3 g., with a mean of 5·8 g. and a standard deviation of 0·43 g.

Range in normal individuals Blood was obtained from 26 young adults, and tensile strength determinations carried out on two or three cuvettes filled from each blood sample, the average for each individual being recorded. The figures ranged from 2·0 g. to 7·3 g. with a

mean of 4·48 and a standard deviation of 1·56 g. This series is too small to give more than a rough indication of the normal variation.

THROMBOTIC STATES Blood was obtained from 30 patients diagnosed as suffering from coronary, or deep vein thrombosis. Most were under treatment with dicoumarol drugs at the time of testing. The tensile strength determinations ranged from 2·9 to 13·0 g., with a mean of 7·24 and a standard deviation of 2·27 g.

There is thus a significant difference between the mean of this series and that of the normal range ($P = < 0.01$).

HAEMOPHILIA It was expected that haemophilic blood clots would be weaker than normal. Blood was obtained from six cases of severe haemophilia and though it was slow to coagulate, the clots once formed were, in four cases, abnormally strong, the figures obtained being as follows: 10·4, 22·0, 6·9, 3·5, 20·7, and 10·6 g., the mean for the group being 12·3 g.

DISCUSSION

The instrument described is simple to use and measures the tensile strength of blood clots with reasonable reproducibility. In its present form it is cumbersome, but, should further observations indicate the usefulness of the method, a smaller and more elegant version could be devised. Preliminary results suggest that wide differences in clot tensile strength exist in pathological states, though the significance of this cannot be judged until the factors responsible have been studied. It is possible, for example, that the increased tensile strength observed in cases of thrombosis and haemophilia is merely a reflection of an increased blood fibrinogen concentration. Many other factors, such as temperature during clotting, packed cell volume, number of platelets, age of clot, contact with glass and other surfaces, and the effect of therapeutic anticoagulants require investigation. As a further application the method might be used to measure the progress of fibrinolysis, and it could be adapted to measure the adhesion of clots to surfaces of different physical or chemical composition, giving information of possible relevance to the understanding of haemostasis, thrombosis, and embolism.

We are grateful to Mr. A. Lord and Mr. T. Strange for their practical help in constructing the tensiometer.

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An automatic staining machine for blood and marrow films on slides

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An automatic staining machine for blood films on coverslips was designed by Davidson, Bareham, Kitchen, and Pegg (1958) and later developed commercially by Elliotts Liverpool Ltd. A modification of this machine was made by Elliotts Ltd. for this laboratory for the automatic staining of blood and marrow films on standard 3 in. × 1 in. slides, and it has been in routine use now for nine months with very satisfactory results. No mechanical or electrical faults have occurred.

GENERAL DESCRIPTION OF SLIDE STAINING MACHINE

Overall dimensions are approximately 20 in. × 20 in. × 13 in. high (Fig. 1). A maximum of seven different staining solutions can be used, each being contained in a standard glass trough (4 in. × 3½ in. × 2¾ in. high) and each of 200 ml. in volume. A maximum of 18 slides (3 in. × 1 in. × 1·0 to 1·2 mm. thick), in pairs back to back, can be stained in any one load. The slides are fitted into a simple stainless steel carrier which is hooked on to the undersurface of the plastic covering disc. The disc bears eight hooks so that the carrier can be hooked on to the disc in any of its eight positions.

FIG. 1. *This photograph is of a machine with the aeration supplied to troughs 6 and 7 and not to troughs 5 and 6 as described in the text; also the safety device to prevent double staining as described in the text is not shown.*

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The timing mechanism is similar to that used on the Elliott tissue processor, a slowly rotating metal disc with notches cut around the edge at the required intervals. As each notch comes into position, the slide carrier is transferred from one staining trough to the next. The minimum time interval is one minute on the standard machine (minimum time intervals of 30 seconds can be provided), with longer intervals in one-minute steps, and a maximum staining cycle of 60 minutes. After the slide carrier has been taken through the staining solutions, it is transferred from the last trough in the seventh position to a draining rack in the eighth position and a warning bell rings for about 30 seconds. Normally the machine is then switched off and the slide carrier removed by hand; if the machine should be left switched on, a safety device, operated by a projection on the edge of the timing disc, prevents the slide carrier from starting on a further staining cycle.

The plastic covering disc, when not in motion, rests on the tops of the staining troughs, so limiting evaporation and dust entry.

Davidson *et al.* (1958), using the coverslip staining machine, found that if the buffered washing solution was kept in motion by bubbling air through it, the scum formed in this solution did not adhere to the coverslips. The same beneficial effect of aeration was found on the slide staining machine. Accordingly an air-pump is fitted to deliver a stream of air to two adjacent staining troughs.

ROUTINE USE OF MACHINE FOR STAINING BLOOD AND MARROW FILMS

The staining cycles on the machine as used in this laboratory (Table) are almost exact copies of the previously employed hand procedures, in which smaller staining troughs (3½ in. × 2¾ in. × 1¾ in. high) and 100 ml. volumes of solutions were used. The only difference is that the two one-minute washes with aeration in troughs 5 and 6 take the place of two quick rinses in buffered distilled water in the hand procedures.

The troughs are filled with fresh solutions (200 ml. volumes) at the beginning of each day, an average day's work involving about 80 to 100 blood films and a set of six marrow films. The effect of using the diluted May-Grünwald and Giemsa solutions for two days was tried, but the staining on the second day was rather pale. Normally the timing disc for blood films is kept fitted on the machine; when marrow films are to be stained, the marrow timing disc is easily substituted in a matter of seconds, and to ensure perfect fixation, fresh absolute methyl alochol is put into trough 2 and also into trough 1. To start the machine, the carrier, loaded with slides bearing the air-dried films, is hooked on to the appropriate hook on the covering disc and lowered into trough 2 for blood films or trough 1 for marrow films, the machine switched on, and the timing disc set at 0 minutes, again in a matter of seconds. Events at the end of a staining cycle have been mentioned above.

TABLE

MACHINE STAINING CYCLES FOR BLOOD AND MARROW FILMS

Trough	*Blood Films*		*Marrow Films*	
	Solution	*Time (min.)*	*Solution*	*Time (min.)*
1	(Not used)	—	Absolute methyl alcohol	10
2	Absolute methyl alcohol	10	Absolute methyl alcohol	10
3	May-Grünwald diluted 1/2[1]	5	May-Grünwald diluted 1/2[1]	5
4	Giemsa diluted 1/10[1]	15	Giemsa diluted 1/10[1]	15
5[2]	Buffered distilled water[3]	1	Buffered distilled water[3]	1
6[2]	Buffered distilled water[3]	1	Buffered distilled water[3]	1
7	Buffered distilled water[3]	10	Buffered distilled water[3]	7

[1]Stock solutions (prepared at the laboratory) diluted with buffered distilled water.
[2]Aeration of the solutions in these two troughs is carried out.
[3]Distilled water buffered to *p*H 6·8 with 150 ml. 0·2M KH_2PO_4 and 71 ml. 0·2N NaOH per 5 litres.

ADVANTAGES OF THE MACHINE

The introduction of this machine has proved to be of great value. Consumption of methyl alcohol, stain solutions, and buffered distilled water has been exactly doubled, but consistent first-class staining of all films is now ensured, regardless of the experience of the person in charge of routine staining, both throughout the day and, if necessary, during the night, as the troughs are left filled overnight. Time and labour are also saved to a considerable extent, as, once the solutions have been renewed at the start of the day, each batch of films requires attention only twice (on to and off the machine) instead of five or six times.

SUMMARY

An automatic staining machine for blood and marrow films is described. It has been in routine use in a busy laboratory for nine months and has proved to be a great asset.

I wish to thank Mr. F. T. Howells of Elliotts Liverpool Ltd., for his cooperation and Mr. J. H. Dunn, F.I.M.L.T., for his assistance.

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The Association of Clinical Pathologists

THE SIXTY-SIXTH GENERAL MEETING was held at the Western Infirmary, Glasgow, from 13 to 15 April 1961. The following are abstracts of the papers read at that meeting.

INTRODUCTORY ACCOUNT OF THE SEX CHROMOSOME ANOMALIES

B. LENNOX (Glasgow) said that sex chromosome anomalies had been more studied than any other, partly because they seem to be especially common, partly because older indirect methods are available to reinforce the newer techniques. One such indirect method is the use of the incidence of sex-linked conditions such as colour blindness to count the X chromosomes. Another is nuclear sexing: under most circumstances the number of X chromosomes is one more than the highest number of sex chromatin bodies per nucleus seen in the buccal smear. The new direct methods depend on counting chromosomes in mitoses produced in tissue culture: the Nowell and Hungerford method, which is most used nowadays, uses the leucocytes of venous blood. The methods used in counting chromosomes and separating them into groups were demonstrated on some typical preparations. The Y chromosome can be identified accurately: the X can usually only be identified as a member of a group, but considering all the evidence, anomalies of the X can nearly always be recognized accurately.

Of possible anomalies those involving the absence of any X chromosome are apparently incompatible with life; XO, XXY, and XXX are common, and a considerable variety of more complex anomalies such as XXXX, XXXY, and mosaics are known. Of the three common anomalies (*a*) XO, with 45 chromosomes in all, is Turner's syndrome (classical Turner's syndrome is usually XO, but there are many variants: mosaicism is common); (*b*) XXY, with 47 chromosomes, is chromatin-positive Klinefelter's syndrome; (*c*) XXX is a newly recognized syndrome usually producing mental defect but little else (the affected women are often fertile) and recognized most easily by the double sex chromatin bodies.

The presumed usual (but not the only) cause of most of these anomalies is non-disjunction, the passage of both members of a chromosome pair into one gamete.

MONGOLISM

KARIN BUCKTON (M.R.C. Radiation Unit, Edinburgh) Chromosomes 13, 14, and possibly 15, 21, and 22 have satellites which may be associated with nucleolar organization. Satellites from one chromosome can form an association with one or a number of other satellites. The stickiness of the satellites on the acrocentric chromosomes may make these autosomes prone to divisional errors. Trisomy of chromosome 13 has been described by Patau *et al.* (1960). Trisomy of chromosome 21 is associated with mongolism and was described by Lejeune *et al.* (1959) and Jacobs *et al.* (1959). The formation of the cell with an additional autosome probably occurs through non-disjunction during gametogenesis.

Penrose has shown that as the maternal age increases, the probability of giving birth to a mongol is greater; but in cases of familial mongolism, the maternal ages are much lower. Other genetic factors may be involved in these families.

Not all mongols have 47 chromosomes. The third chromosome 21 can become translocated onto another chromosome, giving 46 chromosomes. Cases with a translocation between a chromosome 21 and one chromosome in the group 13 to 15 or another 21 or 22 have been reported. It has been possible, particularly in some families with more than one mongol, to trace the translocated chromosome to one of the parents and other apparently normal sibs in their sibship. Not all children born to a parent who carries the translocated chromosome will be mongols but the probability is much increased.

The demonstration of mongol mosaics is of interest. The distribution of 47 and 46 cells could explain why one case (unpublished) of mongol mosaicism has most of the features of mongolism while another case described by Dr. Clarke (personal communication) does not. Mosaicism might be the explanation for some of the unusually intelligent mongols.

It is known that mongols have an increased liability to develop leukaemia and the occurrence of the two conditions in one sibship is known where the mother and at least one of the mongols has a '15'/21 translocated chromosome.

LEUKAEMIA

A. G. BAIKIE (M.R.C. Radiation Unit, Edinburgh) In acute leukaemia in adults, chromosome abnormalities have been found in about one-third of the cases studied. These abnormalities include changes in chromosome number and morphology. In no two cases has the same abnormality been demonstrated. The real incidence of chromosome changes in acute leukaemia may be very much higher than indicated by these results. The results of simultaneous culture of blood and bone marrow have suggested that some negative findings may be due to the failure of leukaemic cells to divide in culture so that they are outgrown by normal cells present in the original sample.

In contrast the abnormal chromosome in chronic myeloid leukaemia (the Philadelphia chromosome) is an

apparently specific abnormality and may be found in most instances of the disease. The minority of cases of chronic myeloid leukaemia in which the Ph^1 chromosome is not found has yet to be defined but may include instances of the disease which run a relatively benign course but are not otherwise atypical. Comparison of the frequency of occurrence of the Ph^1 chromosome in treated and untreated cases suggests that cells carrying the Ph^1 chromosome may be more sensitive to the effects of both *x* rays and busulphan. Studies of instances of acute leukaemic transformation in chronic myeloid leukaemia show the frequent emergence at this stage of a second cell line distinguished by an abnormal chromosome member. It seems likely that the presence of the Ph^1 chromosome in a cell makes that cell especially liable to further genetic change.

The Ph^1 chromosome probably arises as a partial deletion of one of the pair of chromosome No. 21. It is likely that this is the same chromosome for which mongols are trisomic. Increased liability of mongols to develop acute leukaemia is well known. Apart from leukaemia, mongols are known to have a morphological abnormality of the neutrophil polymorphs. Consideration of these facts led the Edinburgh group to advance the hypothesis that chromosome No. 21 may normally carry loci concerned in leucopoiesis.

INTRODUCTION AND SPREAD OF A TYPE 81 STAPHYLOCOCCUS IN A GENERAL HOSPITAL

C. A. GREEN AND I. A. PORTER (Newcastle) An antibiotic-resistant strain of *Staphylococcus aureus* was responsible for an outbreak of infection which involved 61 individuals over a period of 15 months in the medical and surgical wards of a general hospital. The strain of staphylococcus was lysed by phage 81 only when it was employed at a thousand times its routine test dilution (1,000 × R.T.D.).

The transfer of a patient from an infected ward to a previously uninfected ward was an important means of spreading the infection in the hospital.

The development of skin lesions and the infection of surgical wounds were the most common manifestations of this outbreak but pulmonary and urinary infections also occurred and were more dangerous.

Detailed investigations in one ward revealed that the infected patient and the established nasal carrier of staphylococci were the most important sources of infection in the ward. It appeared that in the prevention of staphylococcal infection two manoeuvres were of prime importance, *viz.*, the isolation of the infected patient and the prevention of nasal carriage.

It is believed that this outbreak of staphylococcal infection was brought to an end by the isolation of infected patients and by instituting certain minor changes in the wound dressing techniques.

COAGULASE AS AN IMMUNIZING AGENT AGAINST STAPHYLOCOCCAL INFECTIONS

IWO R. W. LOMINSKI, D. D. SMITH, A. C. SCOTT (Glasgow), and G. TURNER (Beckenham) Coagulase has been shown to act as an aggressin (Smith, Hale, and Smith, 1947). Antibodies to coagulase, present in the sera of many healthy people and absent from the sera of those suffering from septicaemic staphylococcal infection (Lominski and Roberts, 1946), passively protect rabbits against experimental staphylococcal infection (Lominski, 1949). Coagulase also clots *in vivo* (Smith and Johnstone, 1958). Attempts to immunize against staphylococci with coagulase have been only partly successful (Rammelkamp Hezebicks, and Dingle, 1950; Duthie and Lorenz, 1952; Boake, 1956). Rabbits were immunized with alum-adsorbed, alpha and gamma toxin-free coagulase derived from rough variants (Smith, Morrison, and Lominski, 1952) prepared at the Wellcome Laboratories. Rabbits immunized with coagulase from phage group III staphylococci developed antibodies to group III coagulase and were immune to challenge with group III strains. Group I coagulase also provoked an antibody response but protection against group I strains was poor.

In cross-protection experiments some immunity was produced which could not be correlated with the development of antibodies to coagulase and it is suggested that another antigen is associated with coagulase in producing immunity to staphylococcal infection.

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CARBON MONOXIDE POISONING

E. N. DAVEY (Gloucester) reported two unusual cases of carbon monoxide poisoning in which the victims were found in their bedrooms, no gas burners being in the rooms.

In the first case an elderly man of some substance visited some friends from time to time in a house which had been renovated. In his bedroom the fireplace had been bricked up. On each visit he felt unwell and saw the local doctor. On the last visit he was found dead in bed in the morning. The doctor notified the Coroner and at necropsy over 60% CO was found in the blood. But, said the police, there is no gas within two miles of the place. Prolonged investigation eventually revealed a fault in fitting the flue from an agar cooker, so that the fumes entered the room through the floor under the head of the dead man's bed.

In the second case a young man in bed with his wife was found dead beside her, she having 'slept' late. He too had 60% CO in his blood and no gas in the bedroom. A faulty gas cooker was found in the kitchen and after its use some 7% concentration of CO was at floor level. The flow of air was from the kitchen through the bedroom and up towards a ventilator over the wife's head, so that he got the full concentration and she escaped.

A plea was made for regular inspection of gas equipment by gas boards and for the prohibition of the sale of secondhand gas equipment. Also that building inspectors should be more particular when passing renovations to buildings to ensure no faulty fitting of stoves capable of emitting CO.

THE TECHNIQUE OF WEIGHING THE HEART

A. G. MARSHALL (Wolverhampton) said that the best way of detecting hypertrophy of the right ventricle of the heart was by weighing it. Lewis's (1914) technique is time consuming and a much simpler method for use in routine post-mortem work was described. The technique is inaccurate but by the use of the relative weights of the two ventricles and the whole heart, it has been found to be useful in practice. The septum is weighed with the left ventricle and the fat and coronary vessels are not removed. It is important to remove the whole heart from the thoracic viscera and to ensure that no part of the right ventricle remains attached to the septum. The ratios between the left and right ventricles (L/R), between the whole heart and the right ventricle (T/R), and between the whole heart and the left ventricle (T/L) are calculated. When $L/R \geqslant 3$ and/or $T/R \geqslant 5$ it is suggestive of left ventricular hypertrophy. When $T/L \geqslant L/R$ there is right ventricular hypertrophy. Although it was hoped to avoid defining the weight of the normal heart, it was found that the figures were more useful when its deviation above the normal was taken into account.

A series of 650 necropsies in adults was divided on clinical and pathological grounds into three categories of (1) no vascular disease, (2) systemic hypertension, and (3) pulmonary hypertension. It was found that the values of the ratios which show left and right ventricular hypertrophy correspond closely with the last two groups.

The ratios in three diseases of the lungs were examined and it was found that carcinoma of the lung is not significantly associated with pulmonary hypertension. Pulmonary tubercle and pulmonary embolism, however, are closely associated with it; most of the cases in the latter condition are of massive post-operative embolism. It was claimed that a history of chronic lung disease is therefore an indication for early post-operative anticoagulant therapy.

The method applies equally to both sexes at all ages.

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RELATION OF THE BLOOD LIPIDS TO MYOCARDIAL NECROSIS

J. B. ENTICKNAP (London) said that seven fractions of the blood lipids had been examined in about 800 cadaver sera. All are abnormal in ischaemic heart disease and a regular pattern of differences is seen. They are most abnormal when a coronary arterial thrombus is demonstrated at necropsy but only slightly abnormal if the predominant finding is myocardial necrosis. The lipids in cases with gross myocardial fibrosis are moderately elevated and closely resemble those of arterial disease not affecting the heart. Cases of coronary occlusion by atheroma without thrombus or necrosis most closely resemble coronary thrombosis. In non-ischaemic heart disease the lipids resemble those in death from other causes. It thus appears that the onset of myocardial necrosis may be associated with a fall in blood lipids that were previously elevated. The findings also support the contention that coronary occlusion may cause death in the absence of demonstrable necrosis or thrombosis. Some data from clinical cases are consistent with these necropsy findings.

FIBRIN AND INCLUSION BODIES

A. C. LENDRUM (Dundee) The inclusions of giant-cell pneumonia (Kromayer, 1889) were noted by Dürck (1897) to stain as fibrin; later observers (*e.g.*, Hecht, 1910) were not interested in the inclusions, or even failed to see them. On the basis of modern staining methods, it seems unlikely that these inclusions or those in plasma cells can be fibrin, but the inclusions described by Russell (1890) in carcinoma cells might well be.

Granules staining as fibrin occur in phagocytes in lungs with non-infective deposition of fibrin (Lendrum, 1960); they have also been found in the endothelium of small arteries in a partially infarcted ileum (Reid and Dallachy's, 1958, material) showing elsewhere characteristic fibrinous vasculosis. By failing to regard abnormal acidophilic inclusions as possibly fibrin (*e.g.*, Fisher, Fisher, and Lendrum, 1947), histopathologists may have occasionally implied, even if cautiously they had not embraced, viral complicity.

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HAEMATOLOGICAL FINDINGS AND PROGNOSIS IN LUPUS ERYTHEMATOSUS

E. K. BLACKBURN (Sheffield) Comparisons between clinical and haematological findings before and after a five-year period were made in 57 cases of lupus erythematosus (L.E.). Only the single case of the acute disseminated form succumbed to L.E.

None of the 51 chronic discoid cases developed systemic L.E., but one patient developed generalized discoid changes. Clinical manifestations did not correlate with haematological findings in the chronic discoid group.

Patients with chronic discoid L.E. and haematological abnormalities were not shown to be in greater danger of developing the systemic disease than those without such changes.

(Full details are to be published in the *A.M.A. Archives of Dermatology* with Dr. R. H. Marten as co-author.)

DIAGNOSTIC APPLICATIONS OF FLUORESCENT ANTIBODY

R. C. NAIRN, M. G. MCENTEGART, J. E. FOTHERGILL, and I. B. PORTEOUS (Aberdeen) said that the fluorescent antibody method was rapidly becoming an essential laboratory procedure. Using fluorescent-labelled antisera as histochemical stains, it combines microscopical precision with immunological sensitivity and specificity. Technique is standardized and labelling with either fluorescein isothiocyanate or lissamine rhodamine B (RB 200) is simple and quick; some conjugates can be obtained commercially. Fluorescence microscopes are highly developed and conversion units for conventional microscopes are available.

Diagnostic applications include rapid identification of bacteria, protozoa, fungi, and viruses, and refinements in tracing native tissue antigens may eventually be useful. The method is also valuable for identification of antibody.

In microbiology, slowly growing organisms can be rapidly identified in early culture, *e.g.*, by specific staining of microcolonies. The method is less satisfactory for direct examination but even faecal smears have been used successfully for the immediate diagnosis of pathogenic coliforms. Particularly useful, and superior to the search for Negri bodies, is the histological diagnosis of rabies in infected dogs.

Labelled organ-specific antisera for tracing native antigens may eventually facilitate the diagnosis of precancerous conditions. Results have been promising in studies of colonic polyps.

Identification of serum antibody by the technique is established, *e.g.*, in the diagnosis of primary atypical pneumonia due to Eaton's agent; it is at present the method of choice. The fluorescent treponemal test is becoming more widely used as a confirmatory test for syphilis. The demonstration of anti-nuclear and anti-thyroid auto-antibodies by fluorescent tracing is already routine laboratory procedure.

THYROID CYTOTOXIC FACTOR

R. B. GOUDIE and H. MORAG MCCALLUM (Glasgow) The cytotoxic effect of Hashimoto serum on thyroid epithelial cells in tissue culture described by Pulvertaft *et al.* (1959) has been confirmed. The present investigation shows that the thyroid cytotoxic factor in Hashimoto serum has the properties of a specific auto-antibody. (1) Its electrophoretic mobility is consistent with its being a γ globulin. (2) Complement (C′) is a co-factor required for its action (preparations containing C′3 C′4 C′1 C′2 C′4, and C′1 C′2 C′3 are ineffective co-factors as is C′ absorbed with a C′-fixing rabbit-antiovalbumin precipitate). (3) Not only is it tissue specific; cells from normal and thyrotoxic tissue cultures are susceptible whereas cells from single or multiple thyroid nodules ('adenomata') are only partially susceptible. (4) It can be absorbed specifically by homogenates and microsome preparations of thyrotoxic thyroid but not by homogenates of non-toxic nodular goitre. (5) A cytotoxic serum kills cells from the patient's own thyroid.

With some cytotoxic sera no C′ is fixed with homogenates of thyrotoxic thyroid but C′ is nevertheless required to produce the cytotoxic effect on tissue cultures.

Lack of susceptibility of thyroid nodules to cytotoxic factor is due to cells being deficient in the auto-antigen which elicits and reacts with the cytotoxic factor. Loss of this auto-antigen may contribute to the excessive, persistent, and apparently purposeless hypercellularity of these lesions.

COMPARISON OF DIAGNOSTIC TESTS FOR THYROID ANTIBODIES

J. R. ANDERSON, J. S. BECK, R. B. GOUDIE, and KATHLEEN G. GRAY (Glasgow) The diagnostic use of tests upon serum for thyroid antibodies depends on the association of such antibodies with chronic thyroiditis. In general, the more severe the lesions, the more likely will antibodies be present and the higher their titres. Since minor degrees of thyroiditis are common and of no clinical importance, a useful diagnostic test must be relatively insensitive, positive results indicating severe degrees of chronic thyroiditis. Results with precipitin, tanned red cell agglutination, and complement-fixation tests showed the precipitin test to be at least as good as the other two for diagnostic purposes. Since the precipitin test is also the simplest, requiring neither inactivation nor titration of the serum, it is preferred to the others. The Ouchterlony method has certain advantages over other precipitin tests, but some sera take several days to give a precipitate. The latex aggregation ('TA')[1] test for anti-thyroglobulin was performed upon 400 sera: with 24, the test disagreed with the precipitin test. This is because (1) occasional Hashimoto sera contain precipitin which does not aggregate sensitized latex (nor agglutinate sensitized tanned red cells); (2) the 'TA' test is, in general, more sensitive than the precipitin test; (3) approximately 2% of control sera gave apparently false positives with the 'TA' test, which were not annulled by pre-treatment with thyroglobulin. False positive reactions are virtually eliminated by accepting as positive only sera which aggregate latex at both serum concentrations tested (undiluted and 1 in 20). For diagnostic purposes the precipitin test is slightly superior to the 'TA' test. No serological diagnostic test detects all cases of Hashimoto's disease, nor does a positive result exclude the possibility of other disorders, *e.g.*, thyroid malignancy, accompanying chronic thyroiditis.

[1]Reagent supplied by Hyland Laboratories, Los Angeles, California.

ANAESTHETIC AND POST-OPERATIVE HAZARDS IN RHEUMATOID ARTHRITIS

D. L. GARDNER (Edinburgh) Fifty patients with rheumatoid arthritis dying in hospital during the period 1954-60 have been studied at necropsy. Although cardiovascular, respiratory, neoplastic, and renal diseases accounted for the majority of deaths, six patients (12%) were found to have died following palliative or remedial surgical operations. A survey of these six cases showed the following causes of death: gas gangrene after pinning o

fractured femur; systemic candidiasis after gastrectomy; perforation of unsuspected stomal ulcer while under the influence of hexamethonium given during hip arthroplasty; rheumatoid arthritis of larynx with poor post-operative response to sedation. The last case contrasted with a recent surviving case where the difficulty caused by laryngeal rheumatoid arthritis was overcome by laryngostomy. In two further patients post-operative death was not satisfactorily explained. Both patients died after leaving the theatre in good condition. In one there was incomplete collapse of the lower lobe of one lung.

Anaesthesia and operation in patients with rheumatoid arthritis may be hazardous. The evidence suggests that there may be an unexpectedly poor response to anaesthetic, sedative and hypotensive drugs, perhaps due to systemic amyloidosis. Laryngeal arthritis offers difficulties in intubation which may be anticipated when stridor is detected before anaesthesia. Severe infection may supervene in all these cases in spite of the characteristic hyperplasia of the organs of the reticulo-endothelial system. Finally, sudden post-operative death may remain unexplained.

The need for particular caution in operating on the patient with even clinically mild rheumatoid arthritis is emphasized.

HEPATIC CIRRHOSIS AND THE THYROID

G. SCLARE, A. NICOL, and G. TAYLOR (Manchester) In view of several published reports, both histological and immunological, relating thyroiditis to the presence of hepatic cirrhosis, a study of post-mortem thyroid histology was made in 77 cases of cirrhosis of the liver and in an equal number of control cases, matched for age and sex but with no evidence of thyroid or liver disease. Unexpectedly, the results failed to reveal any significant difference between the two groups in the incidence and severity of lymphocytic infiltration of the thyroid.

A high proportion of the thyroid sections, however, showed an increase in fibrous tissue, unrelated to lymphocytic infiltration or to nodule formation. This fibrotic thyroid lesion was present in 23 cases of cirrhosis of the liver as against eight control cases. The preponderance was greatest in women over 50 years of age; of 26 cases in this group, the lesion was present in 11 cirrhotic cases and in two control cases. When the thyroid fibrosis was recorded in three arbitrary grades, it was found that 12 of the cirrhotic cases but none of the control cases occurred in the two higher grades.

A parallel study was made of sera from patients with no clinical evidence of thyroid disease, comprising 33 cases of cirrhosis of the liver and 200 control cases. Thyroid-specific (tanned cell agglutinating) auto-antibody was significantly increased in the cirrhotic group; the increase was greater in women.

'HYPERERGIC INFLAMMATION' IN THE MUSCULAR COAT OF THE INTESTINE AFTER REMITTENT VASCULAR STASIS

H. B. GOODALL (Dundee) said that the main purpose of this paper was to focus attention on the muscular coat of the intestine as a site of inflammatory and degenerative changes. The case of a young woman was described in whom there was an acute segmental sigmoiditis, affecting mainly the muscularis and its vascular plexus, but leaving the mucosa virtually intact, morphologically suggestive of a hyperergic reaction, but probably due to recurrent torsion. This case serves to emphasize vascular disorders as a cause of segmental enteritis, *e.g.*, after mesenteric embolism or hernial incarceration.

Another distinct morphological change, a peculiar, fusiform, acidophilic degeneration of the smooth muscle may also be present in such ischaemic or inflamed bowel, and in cases of gross intestinal distension, *e.g.*, elderly persons with unexplained progressive paralytic ileus. This change is regarded as a pre-lytic phenomenon.

NON-SPECIFIC JEJUNITIS IN CHILDREN AND ITS RELATION TO GIARDIA LAMBLIA

A. H. CAMERON and K. B. ROGERS (Birmingham) Examination of 80 jejunal biopsies at the Birmingham Children's Hospital had revealed a small group of non-specific jejunitis as well as a major group showing a flattened mucosa characteristic of coeliac disease. There was a high incidence of infestation with *Giardia lamblia* in the group with non-specific jejunitis and the patients' symptoms responded to treatment with mepacrine. In three children with non-specific jejunitis no cause of the enteropathy could be found.

ELECTRON MICROSCOPY OF A CEREBRAL BIOPSY FROM A CASE OF SUBACUTE INCLUSION BODY ENCEPHALITIS

T. H. FLEWETT, B. D. BOWER, and A. H. CAMERON (Birmingham) A biopsy of cerebral cortex was removed from the occipitoparietal region of a 9-year-old boy months after the onset of disease. Light microscopy of stained sections of this showed a transition from comparatively normal cortical structure, through an area in which most surviving neurones were greatly shrunken and basophilic in both nucleus and cytoplasm, to an area in which few surviving neurones were present. By phase-contrast these showed dense nuclei and vaculoated cytoplasm; some showed numerous small intranuclear inclusions. These inclusions were seen by electron microscopy to be finely granular masses of irregular shape and size. In a few of the intranuclear masses, small aggregates of particles about 30 to 40 mμ diameter were observed, but their number and distribution was not such that they could be confidently identified as virus particles.

A CASE OF Q FEVER ENDOCARDITIS

N. G. GRIST and J. E. CRAIK (Glasgow) Q fever endocarditis was confirmed by isolation of *R. burneti* from the diseased aortic valve of a man who died after illness lasting one year. The previous history suggested rheumatism. After a short 'influenzal' illness, his health deteriorated and signs of subacute infective endocarditis appeared. Repeated blood cultures were negative. Complement-fixation tests showed high titres (1/6,400) of antibody to both phase 1 and phase 2 antigens of *R. burneti*. At necropsy, the heart was enlarged with

thickening of both mitral and aortic valves. Small vegetations were present on the latter and contained microcolonies of rickettsia-like bodies. Similar particles were seen within large reticuloendothelial cells. *R. burneti* was isolated from aortic valve and in lesser amounts from liver and kidney but not from other tissues examined. Rickettsiae were not seen in sections of liver or other organs, but morphologically similar particles were seen in a few cells of renal tubules. The kidney showed changes of mild membranous glomerulonephritis.

Q fever endocarditis shows a general resemblance to subacute bacterial endocarditis and should be considered in the differential diagnosis of such cases with negative blood cultures.

DEVELOPMENT OF THE PANCREATIC ISLETS IN THE HUMAN FOETUS

PATRICIA ROBB (Dumfries and Galloway Royal Infirmary, Dumfries) Only two papers on the histology of the pancreatic islets in the human foetus had been published since the introduction of reliable methods for differential staining of the α and β cells, and the amount of material forming the basis of these papers was small. The present paper was based on the study of 36 pancreases from foetuses ranging between 10 weeks' gestation and full term (34 g. to 3,600 g.).

Five stages of development were observed. These overlapped, so that the same pancreas contained islets in more than one stage.

STAGE OF BUDDING ISLETS In the earliest pancreases, islet-cells budded off the ducts and formed cords which curved round to enclose a central capillary. α and β cells were already present, though the majority of cells in these young islets were ungranulated.

STAGE OF THE CENTRAL CAPILLARY CLUSTER Developing islets lost their connection with the ducts. The β cells became ranged in pseudo-acini around several central capillaries; α cells and ungranulated cells formed the periphery.

BIPOLAR STAGE Around 16 weeks' gestation, some islets were seen with the β cells grouped at one pole and the α cells at the other. The α-cell pole lay closest to the nearest duct.

MANTLE-ISLET STAGE From the fifth intra-uterine month, islets were found with a solid kernel of β cells and a rim of α cells. The α cells now outnumbered the β cells. These 'mantle-islets' dominated the picture from the sixth month onwards.

STAGE OF MATURE ISLETS From the eighth month, a few islets were observed in which α and β cells intermingled, as they do in the adult islet.

The first three stages had not been described before, and previous workers had not observed islets with the adult structure in the foetal pancreas.

Book reviews

CALCIUM METABOLISM AND THE BONE. By Paul Fourman. (Pp. 325. 37*s*. 6*d*.) Oxford: Blackwell Scientific Publications. 1960.

This is an excellent and much-needed account of the subject. It is quite brief, but surprisingly comprehensive. Although it is written from the point of view of the clinician, it cannot fail to be of interest to the clinical pathologist, for much of its contents relates to biochemical investigations and their interpretation. The book is divided into three sections. The first consists of a general discussion of bone physiology and calcium metabolism. The second deals with what are usually considered as primary disorders of calcium metabolism and bone, *i.e.*, osteoporosis, rickets and osteomalacia, hyperparathyroidism and hypoparathyroidism, while the third is concerned with the effect of renal disease on bone and on the metabolism of calcium and phosphorus.

The chapter on laboratory investigations undertaken in cases of bone disease is likely to be the most useful to clinical pathologists. It includes discussion of the usual biochemical investigations, the use of bone biopsy in the diagnosis of osteomalacia and hyperparathyroidism, and the 'calcium infusion test'. An appendix gives practical information regarding the determination of urinary excretion of calcium, the calcium infusion test, the tubular reabsorption of phosphate, and the Ellsworth-Howard test for hypoparathyroidism: unfortunately, there is hardly any information on, or references to, dependable technical methods for the majority of biochemical procedures concerned in the investigation of bone diseases.

Another surprising omission concerns balance studies: although these are likely to be carried out only in specialized laboratories, they are of outstanding importance in connexion with bone diseases, particularly in relation to the evaluation of various therapeutic procedures, and it is disappointing that they are only mentioned briefly. The use of radioactive tracers in the study of bone metabolism and bone disease is another topic to which scant attention is paid. But these are minor criticisms relating to rather specialized topics, and the book is to be warmly recommended to anyone who wishes to have a sound introduction to the biochemical aspects of bone diseases. There is a good bibliography and a useful index.

H. A. SISSONS

EDEMA: MECHANISMS AND MANAGEMENT. A Hahnemann Symposium on Salt and Water Retention. Edited by John H. Moyer and Morton Fuchs. (Pp. xxix + 833; 312 figures. 105*s*.) Philadelphia and London: W. B. Saunders. 1960.

This contains the papers and discussions of a symposium sponsored by the Hahnemann Medical College at some date which is not mentioned. The wide scope is indicated by the totals of 112 contributors and 123 papers, the latter being grouped into eight sections, namely, fluid and electrolyte balance, diuretics, iatrogenic oedema (from steroids), hypertension, toxaemia of pregnancy and premenstrual tension, oedema of renal origin, oedema associated with liver disease, and congestive heart failure. The contributors include some very well-known names such as Elkington, Merrill, Moyer, and Grollman, and the papers generally are authoritative, though not always lucid. This is not a book which anyone could read through, but it contains a mass of information and each paper has numerous references up to 1959. It is thus a very useful volume for physicians who deal with conditions in which fluid retention is liable to occur, and is also quite a valuable reference book for the chemical pathologist.

G. K. MCGOWAN

LECTURES ON HAEMATOLOGY. Edited by F. G. J. Hayhoe. (Pp. ix + 247; illustrated. 60*s*.) London: Cambridge University Press. 1960.

In December 1959, the University Postgraduate Medical School at Cambridge arranged a refresher symposium on haematology for consultants and senior workers, and in this volume Dr. Hayhoe has edited the 13 lectures presented. No claim is made to provide new or unpublished information: rather, this collection of easily readable papers provides readily available references to current trends in a rapidly expanding discipline. Hence the specialist haematologist, conversant with his subject, will find little of immediate value: as the topics discussed will inevitably soon require revision, he may not feel justified in paying a fairly high price for a book shortly to be out of date. On the other hand, the general physician wishing to keep abreast of changes in haematological diagnosis and treatment will find this an excellent bedside book to inspire his clinical approach. Similarly, the wise group pathologist (and the poor man must have wisdom, for he cannot possibly hope to be expert in more than a tithe of his commitments) will do well to invest in this volume; at least he will be enabled to supply intellectual satisfaction to his more importunate clinical colleagues.

The lectures by J. V. Dacie (acquired haemolytic anaemia) and W. M. Davison (iron metabolism and iron deficiency anaemia) present an excellent survey and, no matter how experienced the reader, can usefully be digested and re-read. R. H. Girdwood (megaloblastic anaemia) and J. C. White (haemoglobin variants) summarize the current literature to which each has made a substantial contribution. G. T. Discombe comments on blood transfusion hazards with a characteristic leavening of common sense, and his remarks should be a mandatory reference for the house resident. G. Wetherley-Mein offers a description of the myeloproliferative syndromes,

valuable as a practical working classification as long as it is appreciated that many members of this group are aetiologically distinct.

Current trends in leukaemia are covered by R. Doll (the incidence of leukaemia and environmental factors); F. G. H. Hayhoe (clinical and cytological differentiation of leukaemia); H. E. M. Kay (marrow transplantation); and D. A. G. Galton (chemotherapy). This catholic selection is rounded off by three complementary lectures on coagulation mechanisms in which R. M. Hardisty discusses the haemostatic process, a considerable undertaking achieved in roughly 14 pages of text; G. I. C. Ingram makes comments on disordered blood coagulation with the same commendable brevity and J. F. Ackroyd describes the pathogenesis of purpura.

The underlying motive for such a symposium is very praiseworthy, and it is sensible that the lectures given should be made available for more general reference. For this to be successful, however, publication should follow as closely as possible upon delivery and it is a pity that nine months elapsed before general distribution. The price is high for this kind of book and it would seem preferable to aim at a cheaper and possibly more rapidly produced publication; by their nature, lectures of this sort remain extant for only a short period of time and are seldom of value for reference purposes beyond the half-life of a 'paperback'. One possible economy would be the exclusion of photographic illustrations which do not contribute very much to the text: those reproduced from colour plates in another publication are frankly disappointing.

One can hope for a second symposium in a year or so: it will be interesting to see how markedly the subject matter and emphasis change.

J. L. STAFFORD

PEDIATRIC PATHOLOGY. By D. Stowens. (Pp. xiii + 750; 374 figures. 160*s*.) London: Baillière, Tindall and Cox. 1959.

This 'Pediatric Pathology' is a remarkable book, the attempt of a single author to describe and illustrate comprehensively the pathology, and in particular the morbid anatomy, of the diseases of infants and children. This Dr. Stowens has achieved by making use not only of his own extensive experience, but also of the resources of the American Armed Forces Institute of Pathology, where in recent years a Registry of Pediatric Pathology has been formed. The most striking feature of this book is the excellence of the illustrations, the majority of which are derived from this Institute, those from the Scholz collection being quite outstanding. These illustrations are almost all photomicrographs, and would serve alone to recommend the book to those who, like the author of this review, tend primarily to assess such a text in terms of its practical value in the analysis of the day-to-day problems of histopathology. Dr. Stowens, however, has attempted to produce rather more than a general analysis of paediatric pathology, for, as he notes in his preface, 'the book is replete with hypotheses, new terms, altered classifications and unanswered questions'. This personal approach enlivens the text, but it must be admitted that some of the ideas put forward would be easier to judge if the extensive references given at the end of each chapter were coupled by numbers to specific statements in the text. It is difficult, for example, to assess the author's justification for claiming that many tumours of the adrenal are in reality 'non-chromaffin paragangliomas': or again, for stating that craniopharyngiomas should not be so named unless they exhibit the 'classical elements' of an adamantinoma. It would be of value also, if in future editions the author were to adduce in greater detail the evidence upon which he bases his rather complex theory of neoplasia, and again his firm conviction that 'the umbilical cord' rarely plays 'a primary role in circulatory failure in the fetus'. In future editions too, the proof requires more careful correction. It is perhaps the mark of a pedant to be irritated by such words as 'supercede, accessability, tuber cinerum, neurohypothesis, syncitial, Antischkow, tetrology and supracellular cyst'. On the other hand, the following sentence, which appears in the preface, is difficult to understand; in discussing his own views, the author notes that 'they are merely ideas on subjects about which no ideas have been expressed or those hypotheses about which, largely derived from thoughts gained by observations on adults, do not fit the facts as seen in children.'

These, however, are minor faults in a text of such length. This 'Pediatric Pathology' is essentially a reference book, the value of which lies in its completeness, the clarity of its descriptions, and the excellence of its illustrations. On these scores it will, undoubtedly, prove of the greatest use to paediatricians, paediatric pathologists, and general pathologists alike.

J. C. SLOPER

METHODS OF GEOGRAPHICAL PATHOLOGY. Report of the Study Group convened by The Council for International Organizations of Medical Sciences. Established under the joint auspices of UNESCO and WHO. Edited by Richard Doll. (Pp. 72. 9*s*. 6*d*.) Oxford: Blackwell Scientific Publications. 1959.

Many who regard geographical pathology as a new development will be surprised to learn that the first textbook on this subject was published about 100 years ago. Geographical pathology, which has received much attention during recent years, studies the distribution of certain diseases in different countries and race groups. Such studies originally consisted of surveys of infectious disease and were therefore included under epidemiology. It is now suggested that many advances might result from the pursuit of similar methods in chronic and degenerative diseases.

The Council for the International Organization of Medical Sciences (C.I.O.M.S.) convened a study group of 'persons with broad interest in pathology and clinical medicine as well as experts in the modern techniques of surveys'. This small book is an excellent report of the study group's findings, and has been very ably edited by Dr. Doll. Details are given of the methods used in geographical pathology and guidance given on registration schemes. A valuable chapter deals with conditions which lend themselves particularly to this type of investigation and the book ends with two appendices giving accounts of a survey on normal blood pressure and hypertension

in Formosa, and of a cancer survey in Johannesberg. Dr. Doll, who played a prominent part in the survey of eclampsia and pre-eclampsia given last year at the International Society of Geographical Pathology meeting in London, is to be congratulated on his editorship of this very useful publication. It will do much to stimulate interest in this valuable approach to the study of disease.

GEORGE J. CUNNINGHAM

CONTROLLED CLINICAL TRIALS. Papers delivered at the Conference convened by The Council for International Organizations of Medical Sciences. Organized under the direction of Professor A. Bradford Hill. (Pp. vii + 177; 40 figures. 20*s*.) Oxford: Blackwell Scientific Publications. 1960.

The need for the clinical trial is becoming better appreciated with the increasing number of therapeutic agents available. Although the benefit of some of these substances is clear-cut, there are many in which it is more difficult to assess their worth. In modern times the clinical trial is a scientific method which has replaced the older method of personal observation. The earlier papers delivered at this conference give guidance on the selection of suitable material for these trials and indicate pitfalls which must be avoided. A series of papers deals with trials carried out on such a wide variety of conditions as 'antihistamine drugs in the treatment of the common cold', 'sore throat', 'tuberculosis', and 'malignant disease'. The assessment of the results is very adequately dealt with and the book concludes with two excellent chapters by Sir George Pickering and by the Chairman, Professor A. Bradford Hill. The production of this volume is of a high standard and the publishers are to be congratulated.

GEORGE J. CUNNINGHAM

HISTOPLASMOSIS. Edited by Henry C. Sweany. (Pp. xiv + 538; illustrated. £5 16*s*.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1960.

This book is a comprehensive account of all aspects of histoplasmosis, each chapter being written by one or more contributors with wide experience in that particular field. Such a book could only be American, since all the important information about the disease has been elicited in the endemic areas of that country; many contributors have been responsible for important advances in the knowledge of this subject and may be truly described as authorities.

The original chapter outlines the history of the disease with a dozen photographs of investigators who have contributed to the subject. From then on detailed descriptions of the organism and its growth characteristics, geographical distribution, the epidemiology of the disease, methods of identification, pathogenesis in animals and man, pathology, radiology, clinical types, diagnostic problems, and therapy, follow. Literature references extend to 1958, and there must be little of importance at that time which is omitted from the volume.

The illustrations are numerous and of excellent quality, and the text, with an occasional notable exception, well written and very readable.

The main criticism of the volume is its unnecessary bulk (and price), which has presumably resulted from the unwillingness of the editor to modify the contributions of his distinguished colleagues. Whatever the reason, repetition is considerable, both in the text and in illustrations; and surely the references could have been gathered together at the end instead of after each chapter, so that many appear in full on numerous occasions. All told they occupy over 40 pages and are not even uniformly set out, some being listed alphabetically, others numerically as they occur in the text. Similarly, it would surely be reasonable to decide whether summaries are needed after each chapter or at the end of the book. The book could be two-thirds its present size and not suffer one bit.

Nevertheless, anyone interested in histoplasmosis will find it a mine of information. And even if one is not particularly interested, it is salutory to read an account of a disease which, until recent years, has been largely misdiagnosed or ignored. The story of this disease is a timely reminder, as the editor points out, that the pathological diagnosis of 'chronic granuloma' should be a diagnostic challenge and not, as is so commonly the case, accepted as an answer. Prolonged antibacterial therapy may be expected to increase the prevalence of fungal infection; perusal of this volume will make the reader less likely to overlook this possibility.

B. E. TOMLINSON

EIGHTH INTERNATIONAL CANCER CONGRESS

The eighth international cancer congress will take place in Moscow from 22 to 28 July 1962, under the auspices of the International Union Against Cancer. The Congress will meet at the Moscow State University, and will consider both experimental and clinical aspects of the problem.

The registration fee is 30 U.S. dollars per member, if sent before 1 April 1962.

Applications to read papers will be considered only on condition that both the application and abstract of the paper (not exceeding 250 words) are submitted not later than 1 November 1961.

All information concerning the Congress, as well as enrolment forms and applications to read papers and show cinematograph films, may be obtained from the General Secretary of the Soviet National Organizing Committee, Professor L. Shabad, or the Assistant General Secretary, Dr. N. Perevodchickova, Academy of Medical Sciences of the U.S.S.R., 14, Solyanka, Moscow, U.S.S.R.

LOCUM BUREAU

The locum bureau is still proving useful both for pathologists wishing to be locums and for pathologists requiring locums. A new system for operating the bureau is now in operation. Anyone interested should get in touch with Dr. Anne Gibson, The South London Hospital, Clapham, S.W.4, Telephone number: TULse Hill 1221.

BACK NUMBERS

The Editor has been sent copies of certain back numbers (not necessarily complete volumes). Anybody who wishes to obtain one or more back issues should kindly write to the Editor at The Group Laboratory, St. Mary Abbots Hospital, Kensington, London, W.8.

CORRECTION

Dr. Meynell writes: 'In the paper by O'Sullivan, Fitzgerald, Malins, and Meynell (*J. clin. Path.*, **13**, 527), under the paragraph entitled "Collection of Specimens", the statement at the end "if this was not possible they were stored at −20°C." is wrong and should read "were stored at +4°C." In this work no specimen had been stored. All were dealt with immediately after collection. We have since compared counts obtained following immediate inoculation; those after storing at +4°C. overnight and those after storing at −20°C. overnight. The first two are practically the same. At −20°C. viable counts are quite strikingly reduced. Although −20°C. may be satisfactory for storing bacteria and filtrable viruses it should not be used for viable counts.'

Association of Clinical Pathologists Broadsheets

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. R. B. H. Tierney, Pathological Laboratory, Boutport Street, Barnstaple, N. Devon**, price 1s. each, except nos. 26 and 27. The prices include postage but air mail will be charged extra.

1 The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics. 1952. R. W. FAIRBROTHER.

2 Determination of Sensitivity of *M. tuberculosis* to Streptomycin. 1952. R. L. VOLLUM.

3 The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents. 1953. L. C. NICKOLLS.

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Studies on the folic acid activity of human serum

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SYNOPSIS A method for the measurement of serum folic acid activity is described, which is a modification of previous methods.

The material in serum with activity for *L. casei* is made up of a stable and a labile component. The amount of stable component in normal subjects and patients with megaloblastic anaemia is similar. The amount of labile component varies. In patients with folic acid deficiency none is present; in normal subjects it constitutes between 65 and 94% of the total serum *L. casei* activity. The labile component appears to be an index of folic acid metabolism, and the assay of total serum *L. casei* activity is therefore a valuable method for differentiating patients requiring treatment with folic acid from normal subjects and patients with primary vitamin B_{12} deficiency. Normal subjects had serum folic acid levels from 5·9 to 21·0 mμg./ml. (mean 9·9 mμg./ml. ± 0·3 mμg./ml. S.E.). In patients with megaloblastic anaemia requiring treatment with folic acid, other than megaloblastic anaemia of pregnancy, the levels were less than 4·0 mμg./ml. Patients with uncomplicated pernicious anaemia had levels from 6·0 to 27·0 mμg./ml. (mean 16·6 mμg./ml. ± 1·1 mμg./ml. S.E.). The mean level in this group was higher than in normal subjects, and the highest levels of all were found in patients with subacute combined degeneration of the cord with minimal anaemia (range 14·4 to 36·8 mμg./ml.; mean 24·8 mμg./ml. ± 2·4 mμg./ml. S.E.).

The *L. casei* activity of the labile component is lost during autoclaving or storage at −20°C. This loss can be prevented during autoclaving by using adequate amounts of ascorbic acid in the phosphate buffer used to dilute the serum for assay and by adding ascorbic acid to serum that is to be stored. Moreover, the activity lost during the storage of serum not protected by ascorbic acid could be restored, for periods up to three months, by adding ascorbic acid to this serum before assay.

The folic acid activity of blood and serum has been measured in the past by a number of workers using *Streptococcus faecalis* (Schweigert and Pearson, 1947; Wolff, Drouet, and Karlin, 1949; Toennies and Gallant, 1949; Girdwood, 1953; Nieweg, Faber, de Vries, and Kroese, 1954; Condit and Grob, 1958; and Cox, Meynell, Cooke, and Gaddie, 1960) and/or *Lactobacillus casei* (Schweigert, 1948; Simpson and Schweigert, 1949; Spray, 1952; and Spray and Witts, 1952) as test organisms. These workers were unable to demonstrate a consistent difference in the levels of folic acid activity of blood or serum in normal subjects, patients with vitamin B_{12} deficiency, and patients with folic acid deficiency, and the results suggested that patients with folic acid deficiency could not be differentiated by measuring the level of folic acid activity in blood or serum.

However, Toennies, Frank, and Gallant (1953) and Toennies, Usdin, and Phillips (1956) showed that the folic acid material in blood was heat-labile and could be preserved for assay either by dialysis at low temperature or by autoclaving in the presence of ascorbic acid. Using these methods they found that the folic acid activity of blood for *L. casei* was 20 to 100 times as high as previously reported, and Grossowicz, Aronovitch, Rachmilewicz, Izak, Sadovsky, and Bercovici (1960) used a modification of this method to measure the blood 'folic acid' levels of mothers and newborn infants at term.

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Baker, Herbert, Frank, Pasher, Hutner, Wasserman, and Sobotka (1959) used a modification of this method to measure the folic acid activity of serum. They found that patients with folic acid deficiency had lower levels of serum folic acid activity than normal subjects or patients with uncomplicated vitamin B_{12} deficiency.

Using the method of Baker *et al.* (1959), we found a considerable overlap between the results in normal subjects and patients with folic acid deficiency. There was also a wide variation in results between different batches. The diagnostic value of the method as described by Baker *et al.* (1959) was therefore of limited value in our hands. It seemed likely that the labile folic acid material was being inadequately protected by the method as we were using it. We therefore studied the factors affecting the preservation of folic acid active material in serum with a view to improving the diagnostic value of the assay. The results of these studies are reported and a modified assay procedure is described.

MATERIALS AND METHODS

BASAL MEDIUM

A modification of the basal medium of Jukes (1955) was used (Table I). A vitamin-free enzymatic casein hydrolysate was used in preference to acid hydrolysed casein (Difco, Oxoid) and salt-free hydrolysed casein (B.B.L.), because both of these preparations gave a precipitate after autoclaving which did not clear on cooling. When the enzymatic casein hydrolysate was used, additional tryptophane was found to be unnecessary.

One batch of a 'dry-mix' medium (B.B.L. folic acid assay medium, special lot 009-607) containing a salt-free acid hydrolysate of casein was also tried. This also gave a precipitate after autoclaving. Nevertheless, it was otherwise satisfactory and would be a convenient medium if small numbers of sera are to be assayed.

This double-strength liquid basal medium was stable when stored in an amber bottle in the refrigerator.

TABLE I

DOUBLE STRENGTH BASAL MEDIUM (*p*H 6·6-6·8)

Reagents	*Concentration (per litre)*	
Enzymatic casein hydrolysate[1]	200	ml.
Adenine sulphate	10	mg.
Guanine hydrochloride	10	mg.
Uracil	10	mg.
Xanthine	20	mg.
L-aspargine monohydrate	600	mg.
L-cysteine hydrochloride	500	mg.
Riboflavin	1	mg.
p-Amino-benzoic acid	2	mg.
Pyridoxine hydrochloride	4	mg.
Thiamine hydrochloride	0·4	mg.
Calcium pantothenate	0·8	mg.
Nicotinic acid	0·8	mg.
Biotin	0·02	mg.
Dextrose (anhydrous)	40	g.
Tween 80 solution (0·5%)	20	ml.
Glutathione (reduced)	5	mg.
Salt solution[2]	10	ml.
Sodium acetate (anhydrous)	40	g.
K_2HPO_4	1	g.
KH_2PO_4	1	g.
$MnSO_4.H_2O$ (added after *p*H adjusted)	200	mg.

[1]Sterile 5% solution of enzyme-hydrolysed vitamin-free casein (Nutritional Biochemicals Corporation, Cleveland, Ohio).
[2]One millilitre contains 40 mg. $MgSO_4.7H_2O$; 2 mg. NaCl; 2 mg. $FeSO_4.7H_2O$; 1·5 mg. $MnSO_4.H_2O$.

MAINTENANCE OF THE STOCK CULTURE

The organism used was *Lactobacillus casei* (ATCC 7469.) This was maintained in dried gelatin discs (Stamp, 1947; Chanarin, Anderson, and Mollin, 1958) and stored in a desiccator over phosphorus pentoxide at room temperature.

Under these conditions the organism remained folic acid dependent and the growth in the control tubes, to which no folic acid was added, was less than 20% of the highest response in the standard tubes.

PREPARATION OF THE INOCULUM

A fresh gelatin disc containing the dried organisms was used for each assay. This was incubated in 10 ml. of Difco microinoculum broth for 30 hours at 37°C. The bacterial suspension was then centrifuged, the supernatant decanted off, and the bacterial cells washed in 20 ml. of single-strength basal medium. Finally, the cells were suspended in 20 ml. of single-strength basal medium to provide an inoculum of standard optical density (approximately 0·04). One drop of this inoculum was delivered to each assay tube with a Pasteur pipette (50 drops/ml.).

The inoculum was plated on a blood agar plate and incubated with the assay batch to detect any contaminants.

COLLECTION OF SERUM SAMPLES

Venous blood was collected from subjects at least two hours after food, and allowed to clot at room temperature for approximately two hours. Serum was separated, care being taken to prevent haemolysis, because it was found that varying degrees of haemolysis increased the folic acid activity from three to ten times that of unhaemolysed serum (unpublished observations). The serum was then stored at −20°C. until assayed.

STANDARD FOLIC ACID SOLUTION

A highly purified crystalline preparation of pteroylglutamic acid was used in preparing the standard curve for each assay, as described by Jukes (1955). Because of the wide range of concentrations of folic acid used in preparing the standard solutions, special safeguards against contamination were taken. Volumetric flasks and pipettes were soaked in chromic-sulphuric fluid, thoroughly rinsed in tap water and finally in distilled water. Moreover, containers were marked and kept for the same concentration of folic acid.

DE-IONIZED WATER

An Elgastat de-ioniser (type B 107) with Elgalite mixed-bed resin (C 403) was used with glass-distilled water.

De-ionized water was used throughout for the preparation of medium and buffer and for the aqueous dilution of serum.

ASSAY PROCEDURE

PREPARATION OF SERUM

Sera were prepared for assay by a modification of previous methods (Toennies *et al.*, 1956; Baker *et al.*, 1959).

Sera were, however, diluted 1:10 with a 0·1 M. phosphate buffer of *p*H 6·1. This buffer concentration has been shown to give the optimum yield of folic acid activity for *L. casei* (Dawbarn, Hine, and Smith, 1958). Various concentrations of ascorbic acid were used in the phosphate buffer (see results); the optimum concentration was found to be 100 mg. %.

The 1:10 serum-buffer solution was autoclaved for two and a half minutes at 15 p.s.i. which was adequate to coagulate the serum proteins. The coagulum was spun down and the clear supernatant diluted 1:4 with de-ionized water, giving a 1:40 dilution of the original serum.

METHOD

The assay was carried out in a total volume of 4 ml. per tube. This volume was selected because, when a serum dilution of 1:40 was used, only 0·5 ml. of serum was required for each assay. It was also convenient for use with the 1 cm. cell of the Unicam colorimeter.

Two millilitres of the 1:40 serum dilution were added to each of four 6 × ¾ in. Pyrex test tubes. Two millilitres of the double-strength basal medium were delivered to each tube by means of an automatic pipetting device, resulting in a final dilution of 1:80 of the original serum. The tubes were then closed with aluminium caps, and autoclaved at 10 p.s.i. for 10 minutes, which provided safe sterilization for this assay.

After cooling, one of the four tubes of each serum specimen was set aside as a blank, and the remaining tubes were placed at random in wooden test-tube racks and inoculated with one drop of the prepared inoculum of *L. casei*. The assay batch was then incubated at 37°C. ± 1°C. for 18 hours.

STANDARD CURVE

A standard curve was included in each assay. Tubes containing 0·0, 0·1, 0·2, 0·4, 0·6, 0·8, 1·0, 1·2, and 1·4 mμg. of folic acid were set up in triplicate, as well as one uninoculated blank tube containing no folic acid (Jukes, 1955). The standard tubes were scattered at random among the other assay tubes.

READING AND CALCULATING RESULTS

Growth was read by measuring turbidity in a Unicam photoelectric colorimeter, using an Ilford 625 filter (λ = 510 to 590 mμ.), and was recorded in optical density units (O.D. × 1,000). Each serum sample was read against its own blank which was used to 'zero' the colorimeter. This procedure eliminated the colour effects of the medium and measured only the turbidity due to the growth of *L. casei*. The same procedure was followed with the standard tubes.

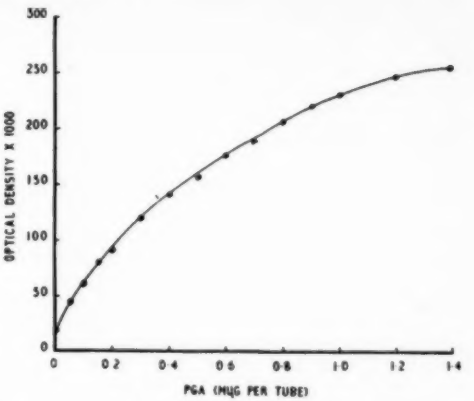

FIG. 1. *A representative standard curve for the* L. casei *assay.*

The growth densities of the folic acid standards were plotted on arithmetic graph paper against the amount of folic acid per tube. A representative standard curve is shown in Fig. 1. The most sensitive range of the curve is between 0·1 and 1·0 mμg. per tube (0·025-0·25 mμg./ml.), which corresponds to a range of serum folic acid activity from 2·0 to 20·0 mμg./ml., when a 1:80 dilution of the original serum is used. If the serum folic acid activity was lower than 2·0 mμg./ml. or greater than 20·0 mμg./ml., the specimen was re-assayed at a lower or higher dilution to give readings on the sensitive part of the curve.

CARE OF GLASSWARE

Scrupulous care of glassware was essential to avoid folic acid contamination.

Test tubes were rinsed in tap water and boiled in a detergent (Pyroneg).[1] They were then well rinsed with distilled water and packed into wire baskets to drain. All tubes were then filled with distilled water, autoclaved at 15 p.s.i. for 15 minutes, emptied, refilled with distilled water, and autoclaved again. The tubes were then dried, closed with aluminium caps, and sterilised at 160°C. for one hour.

Aluminium caps were rinsed in tap water, boiled twice in distilled water, and then dried in a hot air oven.

Pipettes were allowed to stand in detergent and were then washed in running tap water overnight, rinsed through with distilled water, and dried with acetone.

[1] Pyroneg is distributed by Deosan Ltd., 42-46 Weymouth Road, London, W.1.

Flasks, beakers, and measuring cylinders were cleaned with chromic-sulphuric fluid, washed well in running tap water, and finally rinsed with distilled water.

Glassware used in the assay may be kept under ultra-violet light, which results in the photolytic degradation of folic acid (Stokstad, Fordham, and de Grunigen, 1947; Toennies *et al.*, 1953).

SUBJECTS STUDIED

Serum folic acid activity was measured in the following groups of subjects: (1) One hundred normal subjects who were students or members of the staff of the Postgraduate Medical School of London; (2) 42 patients with untreated Addisonian pernicious anaemia, and nine patients with pernicious anaemia in remission following vitamin B_{12} therapy; (3) 22 patients with megaloblastic anaemia of pregnancy and 16 normal pregnant women; (4) 15 patients with untreated idiopathic steatorrhoea, showing evidence of malabsorption with a typically abnormal jejunal biospy appearance; (5) seven patients with nutritional folic acid deficiency. This diagnosis was restricted to patients with a megaloblastic anaemia with a normal serum vitamin B_{12} level, a poor dietary history, no history of taking anticonvulsant drugs, normal absorption of fat, glucose, folic acid, and B_{12}, and no histological or radiological evidence of an intestinal lesion.

RESULTS

THE EFFECT OF ASCORBIC ACID ON THE FOLIC ACID ACTIVITY OF SERUM

EFFECT OF ASCORBATE BUFFER ON THE STANDARD CURVE To determine the effect of ascorbate buffer on the growth of *L. casei* in the standard tubes, a control set of standards was set up containing 2 ml. of an aqueous solution of pteroylglutamic acid (PGA) covering a range of 0 to 1·0 mμg. per tube and 2 ml. of double-strength medium. This was compared with a series of tubes which contained (1) 1·55 ml. of aqueous PGA solution covering the same range of PGA as the standard tubes, (2) 0·45 ml. of ascorbate-phosphate buffer containing either 50, 100, or 150 mg. % of ascorbic acid, and (3) 2 ml. of double-strength medium. This is the amount of buffer contained in the serum tubes during assay. To ensure that conditions in this experiment were the same as in the assay, the ascorbate buffer was autoclaved for two and a half minutes at 15 p.s.i. before it was added to the standard tubes.

The optical density readings at each PGA concentration level are given in Table II. The ascorbate buffer had no effect on the growth of the *L. casei* standards over the range of PGA concentrations used (0-0·25 mμg. per ml.).

TABLE II

EFFECT OF ASCORBIC ACID ON THE GROWTH OF *L. CASEI*

PGA (mμg./tube)	*No Ascorbate Buffer*	*50 mg. % Ascorbate Buffer*	*100 mg. % Ascorbate Buffer*	*150 mg. % Ascorbate Buffer*
0	20[1]	30	25	30
0·1	60	60	60	65
0·15	80	80	85	80
0·2	95	100	90	100
0·3	125	125	120	125
0·4	140	135	135	140
0·5	160	165	160	155
0·6	170	175	175	170
0·7	190	190	185	195
0·8	200	200	205	210
0·9	220	210	215	220
1·0	220	220	230	230

[1]Optical density units = O.D. × 1,000.

EFFECT OF ASCORBIC ACID ON SERUM FOLIC ACID ACTIVITY To study the effect of ascorbic acid on serum folic acid activity, fresh sera from normal controls were prepared for assay, using concentrations from 0 to 150 mg. % of ascorbic acid in the phosphate buffer. The results are given in Table III.

TABLE III

EFFECT OF ASCORBIC ACID ON THE FOLIC ACID ACTIVITY OF SEVEN NORMAL SERA[1]

Sera	*Ascorbic Acid Concentration in Buffer*			
	No Ascorbic Acid	*50 mg. per 100 ml.*	*100 mg. per 100 ml.*	*150 mg. per 100 ml.*
1	4·0	9·0	12·6	12·6
2	1·6	4·0	9·6	9·6
3	2·0	11·2	16·8	16·0
4	2·8	9·0	8·0	7·2
5	3·2	6·0	9·6	8·0
6	4·2	16·8	16·0	16·0
7	4·2	8·4	12·6	10·6
Difference of means		6·1	3·0	0·8
t		4·134	3·109	1·200
P		<0·01 and >0·001	<0·5 and >0·2	<0·3 and >0·2

[1]Results expressed in mμg./ml.

In the absence of ascorbic acid, serum folic acid activity ranged from 1·6 to 4·2 mμg./ml. When ascorbic acid was added to the phosphate buffer the serum folic acid activity increased significantly. A constant level of serum folic acid activity was obtained when the ascorbic acid concentration in the buffer was 100 mg. %.

The importance of using the optimal concentration of ascorbic acid in the buffer is shown in Table IV. This compares the folic acid levels obtained when

TABLE IV

EFFECT OF HEAT ON SERUM FOLIC ACID ACTIVITY OF NORMAL SUBJECTS AND PATIENTS WITH FOLIC ACID DEFICIENCY

Condition	*Sera*	*Stable (S)*	*Total (T)*	*Labile (T–S)*	$\frac{L}{T} \times 100\%$
Normal subjects	1	4·0	12·6	8·6	68
	2	1·6	9·6	8·0	83
	3	2·0	16·8	14·8	94
	4	2·8	8·0	5·2	65
	5	3·2	9·6	6·4	67
	6	2·0	9·0	7·0	78
	7	1·8	9·6	7·8	81
	8	2·2	8·0	5·8	73
	9	1·0	10·0	9·0	90
	10	1·2	8·0	6·8	85
	11	2·0	5·9	3·9	66
	12	1·6	7·6	6·0	79
	13	1·4	11·4	10·0	88
	14	2·2	7·0	4·8	69
	15	4·0	18·0	14·0	78
	Mean	2·2	10·1	7·9	77·6
	S.E.	0·2	0·5	0·8	6·1
Folic acid deficiency	16	2·8	3·0		
	17	2·0	2·4		
	18	2·4	2·5		
	19	2·6	3·2		
	20	2·6	3·5		
	21	2·6	2·6		
	Mean	2·5	2·9		
	S.E.	0·1	0·2		

sera from 15 normal subjects and six patients with folic acid deficiency were assayed with and without ascorbic acid in the buffer.

In the absence of ascorbic acid there was no significant difference ($P > 0{\cdot}4$) between the levels obtained in normal controls and cases of folic acid deficiency. However, when the optimum concentration of ascorbic acid was used in the phosphate buffer there was a highly significant increase ($P < 0{\cdot}001$) in the serum folic acid activity of the normal control group, but no significant change ($P > 0{\cdot}6$) in the serum folic acid activity of the folic acid-deficient group.

EFFECT OF STORAGE AT $-20°$C. ON SERUM FOLIC ACID ACTIVITY To study the effect of storage at $-20°$C. on serum folic acid activity, sera from normal control subjects were assayed within 48 hours of being taken and again one week, three weeks, and three months later. The results are given in Table V. This shows that after one week the residual serum folic acid activity was only 75% of its original value, after three weeks 67%, and after three months 64%.

There was no significant difference in the folic acid activity of sera from six folic acid-deficient patients after three months' storage.

EFFECT OF STORAGE WITH ASCORBIC ACID ON SERUM FOLIC ACID ACTIVITY Fresh serum from normal subjects was divided into two portions. To one portion was added either dry ascorbic acid (5 mg. per ml. of serum) or an equal volume of 0·1 M phosphate buffer of *p*H 7·4, containing 300 mg. % of ascorbic acid. The treated and untreated samples were assayed by the standard procedure using a concentration of 100 mg.% of ascorbic acid in the buffer within 48 hours of being collected, then all samples were stored at $-20°$C. for three months and re-assayed. The results of these experiments are given in Table VI.

Adding ascorbic acid to fresh serum prevented the loss of folic acid activity during storage, there being no significant difference between the folic acid activity of fresh and stored serum ($0{\cdot}4 > P > 0{\cdot}3$). However, the added ascorbic acid had no effect on the folic acid activity of fresh serum ($0{\cdot}8 > P > 0{\cdot}7$).

Some sera were stored with higher concentrations of ascorbic acid. It was found that the protective effect of ascorbic acid was the same with concentrations between 5 and 15 mg. per ml. of serum, but higher concentrations interfered with the preparation of sera for assay by lowering the *p*H and thereby preventing the coagulation of serum proteins during autoclaving.

EFFECT OF ASCORBIC ACID ON FOLIC ACID ACTIVITY OF STORED SERUM To determine whether the lost folic acid activity of stored serum could be restored by the subsequent addition of relatively large amounts of

TABLE V

LOSS OF SERUM FOLIC ACID ACTIVITY DURING STORAGE AT $-20°$C.

Classification	*Number of Subjects*	*Serum Folic Acid Activity (mean ± S.E. mμg./ml.)*				*Percentage of Initial Folic Acid Activity Remaining (mean ± S.E.%)*	*Significance of the Differences P*
		Fresh	*One Week*	*Three Weeks*	*Three Months*		
Normal controls	30	9·7 ± 0·7	7·4 ± 0·5	—	—	75·8 ± 2·6	<0·001
	10	10·6 ± 1·2	—	7·1 ± 0·9	—	67·2 ± 3·7	<0·05, >0·02
	23	10·3 ± 0·5	—	—	6·5 ± 0·3	64·1 ± 3·4	<0·001
Severe folic acid deficiency	6	2·8 ± 0·2	—	—	2·2 ± 0·2	—	>0·6

TABLE VI

EFFECT OF ASCORBIC ACID ON THE FOLIC ACID ACTIVITY OF SERUM DURING STORAGE AT −20°C.

Sera	Fresh	Three Months Old		
	No Ascorbic Acid	With Ascorbic Acid	No Ascorbic Acid	With Ascorbic Acid
1 *Adding Dry Ascorbic Acid to Fresh Serum (5mg. per ml.)*				
1	13·2	13·5	7·4	14·0
2	16·0	15·5	8·4	16·0
3	6·3	6·7	3·6	6·8
4	8·4	8·0	5·8	8·0
5	7·5	7·0	5·0	8·0
6	8·0	9·0	6·6	8·0
7	10·8	9·5	7·2	10·0
8	10·0	9·8	5·2	10·8
9	8·3	7·8	4·4	8·4
10	10·5	10·3	6·0	12·8
11	11·0	12·5	6·8	13·6
12	10·5	9·8	6·4	10·4
13	9·3	9·6	5·2	10·4
14	10·8	10·0	7·4	10·8
15	10·3	9·8	7·2	12·8
16	11·3	11·3	7·4	12·8
2 *Adding Equal Volume of Ascorbate-phosphate Buffer (300 mg. % ascorbic acid) to Fresh Serum*				
17	10·5	10·0	7·5	11·1
18	14·0	18·0	8·8	16·0
19	6·4	7·0	4·4	6·6
20	8·5	8·0	5·7	8·0
21	10·4	12·0	8·6	12·8
22	12·0	13·2	8·2	13·4
23	13·2	13·5	7·2	12·4

ascorbic acid, dry ascorbic acid (5 mg./ml. of serum) was added to a portion of the untreated serum that had been stored at −20°C. for three months (see Table VI), and the folic acid activity was assayed by the standard procedure. The folic acid activity of sera treated in this way is compared in Table VII with the folic acid activity of fresh serum and serum stored alone at −20°C. for three months. There was no significant difference ($0{\cdot}4 > P > 0{\cdot}3$) between the levels obtained in fresh serum and serum treated in this way.

TABLE VII

EFFECT OF ADDING ASCORBIC ACID TO SERA STORED FOR THREE MONTHS AT −20°C. IMMEDIATELY BEFORE ASSAY

Sera	Fresh	After Storage for Three Months at −20°C.		
		Stored without Ascorbic Acid	Stored with Ascorbic Acid	Ascorbic Acid Added to Stored Serum Immediately before Assay
1	11·0	5·2	10·4	7·4
2	9·5	6·4	10·4	10·4
3	10·5	7·4	10·4	10·0
4	14·0	10·4	16·0	14·0
5	9·1	7·4	10·8	8·4
6	13·0	7·2	12·8	10·8
7	12·0	7·4	12·8	12·0
8	13·2	6·8	13·6	11·2

ACCURACY OF THE METHOD

The following experiments were carried out to determine the accuracy of the standard method described.

RECOVERY OF PGA ADDED TO POOLED 'LOW' SERUM Because the nature of serum folic acid activity is unknown, recovery experiments cannot strictly be carried out. However, the results (Table VIII) show that by this method there is a good recovery of PGA added to serum. When 10 mμg./ml. of PGA was added to pooled 'low' serum the mean recovery was 113·4% ± 4·7% S.E., and when 20 mμg./ml. was added the mean recovery was 99·0% ± 4·3% S.E.

VARIATION BETWEEN DIFFERENT ASSAYS This was studied by repeated assays on the same two 'control' sera in eight consecutive assays. The results (Table IX), show that with the 'high' control the coefficient of variation was 10·8% and with the 'low' control 18·2%.

TABLE VIII

RECOVERY EXPERIMENTS

Assay No.	L. casei *Activity (mμg./ml.)*				
	Pooled Low Serum	Pooled Low Plus 10 mμg./ml. PGA	Recovery of Added PGA (%)	Pooled Low Plus 20 mμg./ml. PGA	Recovery of Added PGA (%)
1	2·6	15·2	126	21·6	95
2	2·0	10·8	88	18·0	80
3	2·8	13·2	104	24·0	106
4	1·8	14·4	126	19·2	87
5	2·0	13·0	110	23·0	105
6	2·4	14·8	124	24·0	108
7	2·5	13·5	110	26·0	117·5
8	2·5	14·4	119	21·2	93·5
Mean	2·3	13·7	113·4	22·1	99·0
S.E.	0·1	0·5	4·7	0·9	4·3

TABLE IX

INTER-ASSAY VARIATION

Assay No.	*'High' Control (mμg. per ml.)*	*'Low' Control (mμg. per ml.)*
1	14·8	2·6
2	11·0	1·6
3	12·8	2·4
4	16·0	2·0
5	14·8	2·3
6	14·2	1·7
7	13·2	2·0
8	14·6	2·8
Mean	13·9	2·2
S.E. of mean	±0·5	±0·2
Coefficient of variation	10·8%	18·2%

INDEPENDENT DOUBLE DETERMINATIONS These were carried out (*a*) in the same assay on 47 different sera, and (*b*) in different assays on 75 different sera.

At the 5% level of significance, the maximum variation from the mean of two independent determinations on the same sample in the same assay was ± 16%, and in different assays ± 20%.

We have also shown that there is no significant day-to-day variation in the serum folic acid activity of the same subject. Sera collected from 26 subjects on two consecutive days were assayed in the same batch. The folic acid levels ranged from 2·0 to 25·0 mμg./ml., and there was no significant difference between the duplicate samples in each case.

In less than 1% of cases the variation between the folic acid activity of two consecutive daily serum samples from the same patient greatly exceeded the expected error of the method. In these cases this wide discrepancy between the results was due to exogenous PGA contamination. This was shown in two ways. The contaminated specimen was re-assayed, (*a*) using *Streptococcus faecalis* as the assay organism, which responds to PGA, but not to serum folic acid material; and (*b*) by the standard procedure for *L. casei* without adding ascorbic acid either to the fresh serum or to the phosphate buffer. Under these conditions serum folic acid activity is destroyed, whereas PGA is not.

SERUM FOLIC ACID ACTIVITY IN NORMAL SUBJECTS AND PATIENTS WITH MEGALOBLASTIC ANAEMIA

The folic acid activity of sera from normal subjects and patients with megaloblastic anaemia measured by this method are given in Table X. Each value recorded is the average of two independent assays on two samples taken on consecutive days.

The levels of serum folic acid activity in normal subjects ranged from 5·9 to 21·0 mμg./ml. The levels in patients with megaloblastic anaemia due to folic acid deficiency associated with idiopathic steatorrhoea and nutritional folic acid deficiency were less than 3·5 mμg./ml.

There was no overlap between the levels obtained in cases of megaloblastic anaemia due to folic acid deficiency and those due to uncomplicated B_{12} deficiency. Of the 42 cases of untreated pernicious anaemia, only one had a folic acid level below the lower limit of the normal range. The folic acid level in this case was 4·0 mμg./ml. and the serum B_{12} level 35 μμg./ml. This patient failed to respond adequately to treatment with vitamin B_{12} alone and required folic acid as well for a full haematological response.

The mean serum folic acid activity in cases of untreated pernicious anaemia was significantly higher ($P < 0.001$) than in normal subjects. The highest levels of all were found in eight cases of subacute combined degeneration of the cord with only minimal anaemia (range 14·4-36·8 mμg./ml.; mean 24·8 mμg./ml. ± 2·4 mμg./ml. S.E.). Following treatment with B_{12} the serum folic acid activity fell and there was no significant difference ($0.4 > P > 0.3$) between the levels of normal subjects and

TABLE X

SERUM *L. CASEI* ACTIVITY IN VARIOUS CONDITIONS

Diagnosis	*No. of Cases*	*Serum* L. casei *Activity (mμg./ml.)*		*Serum* B_{12} *(μμg./ml.*[1]*)*
		Range	*Mean ± S.E.*	
Normal subjects	100	5·9 – 21·0	9·9 ± 0·3	140 – 900
Pernicious anaemia				
Untreated	42	4·0 – 27·0	16·6 ± 1·1	5 – 100
Treated	9	5·0 – 14·0	9·0 ± 0·9	230 – 1175
Subacute combined degeneration of the cord	8	14·4 – 36·8	24·8 ± 2·4	25 – 60
Folic acid deficiency	22			
Idiopathic steatorrhoea	15	0·7 – 3·5	1·7 ± 0·2	100 – 360
Nutritional folic acid deficiency	7	0·6 – 3·5	2·1 ± 0·4	90 – 390
Pregnancy				
Normal	16	7·0 – 30·0	16·4 ± 2·0	115 – 480
Megaloblastic anaemia	22			
Severe	12	1·3 – 4·3	2·6 ± 0·3	90 – 400
Mild	10	4·0 – 7·0	5·0 ± 0·3	145 – 470

[1] *Euglena gracilis* assay (Hutner, Bach, and Ross, 1956).

cases of pernicious anaemia in remission following treatment with vitamin B_{12}.

The mean serum folic acid level in normal pregnant women was significantly higher ($P < 0.001$) than in normal control subjects and not significantly different from the mean level found in cases of untreated pernicious anaemia. In patients with severe megaloblastic anaemia of pregnancy the levels were as low as in cases of severe folic acid deficiency associated with idiopathic steatorrhoea and nutritional folic acid deficiency, but there were also some patients with levels between 4·0 and 7·0 mμg./ml. The bone marrow in these cases was only slightly megaloblastic. However, Table X shows that the mean serum folic acid levels in both of these groups was well below the mean level found in normal pregnant women.

OTHER FACTORS AFFECTING SERUM *L. CASEI* ACTIVITY

The effect of antibiotics on serum *L. casei* activity was studied in three patients. Two of these patients had bronchopneumonia. One was being treated with tetracycline, 250 mg. q.i.d., when the serum sample was taken, and the other with procaine penicillin, 1 million units daily, and streptomycin, 0·5 g. b.d. The third patient, who had puerperal sepsis associated with megaloblastic anaemia, was being treated with chloramphenicol and neomycin, āā 250 mg. q.i.d. Moreover, this patient was also receiving pteroylglutamic acid, 25 mg. daily. Serum from the two patients receiving tetracycline and chloramphenicol and neomycin inhibited the growth of *L. casei* at a 1:80 dilution, the latter despite the fact that the patient was receiving PGA simultaneously. However, serum from the patient receiving penicillin and streptomycin had an *L. casei* activity in the normal range (9·4 mμg./ml.).

The effect of amethopterin was studied in a patient with acute leukaemia, who was being treated with 2·5 mg. of amethopterin on alternate days. Serum collected on the days that amethopterin was given inhibited the growth of *L. casei* at a 1:80 dilution, whereas serum collected on the intervening days had a normal *L. casei* activity (6 mμg./ml.).

DISCUSSION

The results given in this paper suggest that the difficulties we encountered using the assay described by Baker *et al.* (1959) were mainly due to (1) inadequate protection of the labile folic acid-like material during autoclaving and (2) loss of this material during storage. Our observations suggest that these losses can be prevented by increasing the amount of ascorbic acid in the phosphate buffer and by adding ascorbic acid to serum that is to be stored. When these precautions are taken the variation between the results in different assays is slight and there is a clear-cut differentiation between the results in normal subjects and patients with megaloblastic anaemia requiring treatment with folic acid (Table X).

PROTECTION OF LABILE FOLIC ACID MATERIAL DURING AUTOCLAVING

The importance of ascorbic acid and of other reducing agents for protecting labile folic acid activity of blood during assay has been emphasized by Toennies *et al.* (1956). For this purpose they included 50 mg. % of ascorbic acid in their phosphate buffer and the same procedure was followed by Baker *et al.* (1959) in assaying serum. The results in Table III of this paper show that these amounts were inadequate for serum, but that the protection was complete when amounts of 100 mg. % or more were used. Herbert (1961) has also found it necessary to increase the concentration of ascorbic acid in the buffer.

The role of ascorbic acid is uncertain. The added ascorbic acid presumably is required to protect the labile folic acid material during autoclaving (Toennies *et al.*, 1956). During this process ascorbic acid is also destroyed, but about 40% remains so that the final concentration in each assay tube was about 0·1 mg. per ml. (Dawbarn *et al.*, 1958; Waters and Mollin, 1961). Herbert (1961) reports that ascorbic acid *per se* may stimulate the growth of *L. casei* in response to PGA. However, he studied the effect using much larger concentrations of folic acid than are used in the assay and with more ascorbic acid, for he did not autoclave the ascorbate-phosphate buffer before its addition to the assay tubes. The results of this paper suggest that under the conditions of the assay the concentrations of ascorbic acid used had no effect on the growth of *L. casei*.

PROTECTION OF LABILE FOLIC ACID MATERIAL DURING STORAGE OF SERUM

Toennies *et al.* (1953) pointed out that the folic acid activity of whole blood dialysates is rapidly lost if stored at 2°C, but this change did not occur if sera were stored frozen, or with ascorbate-phosphate buffer at 2°C.

The results here show that there was a considerable loss of folic acid activity in serum stored frozen at −20°C but that this loss could be prevented if ascorbic acid was added before the serum was frozen. The apparently 'lost' folic acid activity could be restored by adding ascorbic acid to previously stored serum (Table VII). To do this, larger concentration-

of ascorbic acid appear to be required than are needed to protect the material during autoclaving.

NATURE OF THE *L. CASEI* ACTIVE MATERIAL IN SERUM

The nature of the stable and labile folic acid material in serum is unknown. Although blood contains a number of factors with folic acid activity, neither pteroylglutamic acid nor citrovorum factor are present (Usdin, 1959).

The stable material resembles in certain respects pteroylglutamic acid, for it is active for *L. casei*, *Strep. faecalis*, and *L. citrovorum*, but other folic acid compounds and nucleotides also have similar characteristics. Furthermore, the fact that the concentrations of stable material in the serum of patients with pernicious anaemia and folic acid deficiency are similar to those found in normal subjects suggests that it is unlikely that this material is pteroylglutamic acid.

The labile folic acid factor resembles in some respects a precursor of citrovorum factor. It has been shown that ascorbic acid protects a labile precursor of folinic acid which appears in urine after the ingestion of folic acid (Silverman, Ebaugh, and Gardiner, 1956). However, the labile component in serum is not converted into folinic acid by autoclaving in the presence of ascorbic acid, because the folic acid material in serum has very little growth activity for *Leuconostoc citrovorum* (*Pedicoccus cerevisiae*) or *Streptococcus faecalis* (Toennies *et al.*, 1953; Usdin, 1959; Herbert, 1961; Waters and Mollin, unpublished observations), and Baker *et al.* (1959) and Herbert (1961) have suggested that it may well be a polyglutamate.

The significance of the increase in labile *L. casei* active material in untreated Addisonian pernicious anaemia and normal pregnant women is uncertain. The material presumably accumulates either because it is formed in excessive amounts in these patients or because there is a defect in its utilization. This observation is particularly interesting in view of the fact that injected pteroylglutamic acid is removed more rapidly from the plasma of patients with severe untreated pernicious anaemia than from normal subjects (Chanarin, Mollin, and Anderson, 1958). It was also found by Chanarin, MacGibbon, O'Sullivan, and Mollin (1959) that the mean plasma clearance of injected pteroylglutamic acid was more rapid in normal pregnant women than in normal control subjects.

OTHER MODIFICATIONS OF THE ASSAY PROCEDURE

The method described differs in certain other respects from that described by Baker *et al.* (1959).

1 Enzymatic casein hydrolysate is used instead of salt-free acid hydrolysed casein. This was used because with acid hydrolysed casein some precipitation occurred in the medium during autoclaving and this led to a high blank reading. A similar precipitation was observed with B.B.L. 'dry-mix' medium which, however, was otherwise very satisfactory.

2 The final volume in each assay tube was reduced from 10 ml. to 4 ml., and a final serum dilution of 1/80 was used. As a result of these changes only 0·5 ml. of serum was required for the assay and the range of serum folic acid activity that could be measured accurately was 2·0 to 20·0 mμg./ml., compared with 3·0 to 30·0 mμg./ml. by the method of Baker *et al.* (1959), who used a final serum dilution of 1/100. Cooperman, Luhby, and Avery (1960) have recently described a method of assay with *L. casei* in which the assay volume was reduced to 2 ml.; however, the overall sensitivity of this method was reduced by the very high serum dilutions which they used (1/250 to 1/1,000); the lower limit of serum folic acid activity that could be measured accurately at their lowest dilution being 3·75 mμg./ml.

3 INCUBATION BEFORE ASSAY In the method described by Toennies *et al.* (1956) blood, after dilution with the ascorbate-phosphate buffer (50 mg. % ascorbic acid), was incubated for 90 minutes at 38°C. before autoclaving, and this procedure was followed by Baker *et al.* (1959). This was found to be unnecessary if the serum was assayed within 48 hours of collection or if it had been stored with added ascorbic acid or if the lost folic acid activity of stored serum was restored by the addition of relatively large amounts of ascorbic acid. Herbert (1961) also found that incubation of the serum-ascorbic acid-buffer solution did not increase the folic acid activity of (presumably) fresh serum.

CLINICAL VALUE OF THE ASSAY

The results in Table X confirm the claim of Baker *et al.* (1959) and of Herbert, Baker, Frank, Pasher, Sobotka, and Wasserman (1960) that the *L. casei* assay is a valuable means of differentiating patients with megaloblastic anaemia requiring treatment with folic acid from patients with vitamin B_{12} deficiency. In patients with megaloblastic anaemia due to idiopathic steatorrhoea or dietary folic acid deficiency, the concentrations were invariably below the normal range. Similar results were also found in patients with severe to moderate megaloblastic anaemia of pregnancy. However, borderline levels were found in some patients with moderate to mild megaloblastic anaemia of pregnancy, but these were

below the levels found in normal pregnant women. Borderline levels were also found in one patient with pernicious anaemia who failed to respond adequately to treatment with B_{12} alone. In cases of uncomplicated B_{12} deficiency the levels were higher than normal and the highest levels of all were found in cases of subacute combined degeneration of the cord with minimal anaemia.

We have also observed that, when treatment with folic acid is stopped in patients with folic acid deficiency, megaloblastosis will reappear in the marrow while the serum *L. casei* concentration is still within the normal range (Waters and Mollin, unpublished observations). This suggests that the level of *L. casei* active material in serum is in part a function of the duration of the folic acid deficiency.

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Conventional voltage electrophoresis for formiminoglutamic-acid determination in folic acid deficiency

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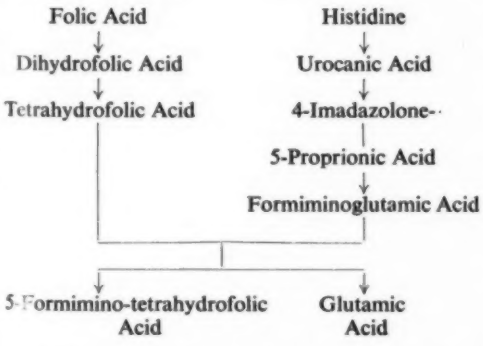
SYNOPSIS A new method for the determination of urinary formiminoglutamic acid (FIGLU) using conventional electrophoresis at 200 to 500 v. on cellulose acetate strips is reported. Experience in 166 determinations on 137 patients shows the method to be a simple, practical, and apparently sensitive one for the determination of FIGLU in the urine.

Results of the application of the measurement of urinary FIGLU with histidine loading as a test for folic acid deficiency are also reported.

In 1951, Bakerman, Silverman, and Daft detected a precursor of glutamic acid in the urine of folic acid-deficient rats. This material was found to be formiminoglutamic acid (FIGLU) and was shown to be an intermediate product of histidine metabolism (Borek and Waelsch, 1953; Tabor, Silverman, Mehler, Daft, and Bauer, 1953; Tabor and Mehler, 1954; Seegmiller, Silverman, Tabor, and Mehler, 1954). It was also found that for the further metabolism of FIGLU to glutamic acid, folic acid in the form of tetrahydrofolic acid is required (Miller and Waelsch, 1956 and 1957; Tabor and Rabinowitz, 1956). The metabolism of histidine and the relationship to folic acid is as follows:



Therefore in the presence of folic acid deficiency FIGLU is not further metabolized to glutamic acid and appears in increased amounts in the urine. Increased urinary FIGLU has been demonstrated in patients with amethopterin-treated leukaemia (Broquist, 1956; Hiatt, Goldstein, and Tabor, 1958; Broquist and Luhby, 1959) and in megaloblastic anaemias due to folic acid deficiency (Broquist and Luhby, 1959; Luhby, Cooperman, and Teller, 1959a and 1959b; Spray and Witts, 1959). The use of histidine loading increases the sensitivity of the test (Luhby, *et al.*, 1959b).

Although the measurement of urinary FIGLU was considered to be a sensitive test for folic acid deficiency, clinical application has been limited by the complexities of the methods required for the determination of FIGLU. These methods include a microbiological assay using *Lactobacillus arabinosus* (Silverman, Gardiner, and Bakerman, 1952; Broquist, 1956), an enzymatic method (Tabor and Wyngarden, 1958), and a combined enzymatic-microbiological technique (Silverman, Gardiner, and Condit, 1958). Paper chromatography (Silverman *et al.*, 1952) has also been used, but was not regarded to be sufficiently sensitive. Knowles, Prankerd, and Westall (1960) introduced the use of high-voltage electrophoresis for FIGLU determination but this method requires expensive equipment and the use of 2,000-6,000 volts.

Recently we introduced the use of conventional voltage electrophoresis on cellulose acetate strips as a simple and practical method for the determination of FIGLU (Kohn, Mollin, and Rosenbach, 1961). We now wish to report the method in detail and its application as a test for folic acid deficiency.

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METHOD

PRINCIPLE Formiminoglutamic acid (FIGLU) in the urine is separated by means of small-scale cellulose acetate electrophoresis (Kohn, 1959, 1960) in a pyridine-acetic acid buffer. It is then demonstrated by staining with ninhydrin after exposure to ammonia, which converts FIGLU to glutamic acid. Two parallel aliquots of the patient's urine (after administration of histidine) are run at the same time and under the same conditions, together with aliquots of a 'marker' urine containing a known quantity of FIGLU. By comparing the corresponding spots on the ammoniated strip with those on the non-ammoniated one the presence of FIGLU in the urine can be detected and its approximate concentration estimated. With a current of 200 v. the mobilities of FIGLU and glutamic acid are almost identical and their spots overlap to a great extent. The interpretation of the results is, however, quite easy due to the fact that FIGLU can only be demonstrated on the ammoniated strip.

Using higher voltages, 400-500 v., a satisfactory separation of FIGLU from glutamic acid can be achieved without overlapping. The choice of the method depends mainly on the degree of experience and whether a suitable power pack is available. For routine purposes, however, 200 v. is recommended as being simpler and more reliable.

MATERIALS

SUPPORTING MEDIUM Cellulose acetate strips (Oxo Ltd.) are supplied in 36 × 5 cm. size. For the FIGLU technique use 12 × 5 cm. strips.

BUFFER SOLUTION Pyridine-acetic acid buffer *p*H 5·3 is made up of pyridine, 50 ml., glacial acetic acid, 20 ml., distilled water, 4 litres.

LOCATION REAGENT This is a ninhydrin solution in ethanol ether. Dissolve 0·2 g. ninhydrin in 6 ml. of ethanol and make up to 100 ml. with diethyl ether. This ninhydrin reagent appears to be particularly suitable for cellulose acetate separation. It is recommended that traces of ammonia be removed from the ninhydrin reagent; this can be done by adding a cation-exchange resin in hydrogen form (Jacobs, 1960).

AMMONIA 0·88.

FIGLU MARKER This can be prepared by adding a known amount of FIGLU to acidified normal urine. The final concentration of FIGLU in this marker urine can be made into a series covering a range from 100 to 1,000 μg. per ml. Pure FIGLU, however, is difficult to prepare and it is not yet commercially available. It is much simpler to use as a marker a FIGLU-positive urine from a patient with folic acid deficiency. If the FIGLU concentration of such marker is once estimated it can be used for approximate quantitative determinations. The FIGLU concentration of acidified urine, to which a few thymol crystals are added, does not change for many months if kept at 4°C.

GLASS CAPILLARY AUTOMATIC PIPETTES These are most useful, and can be easily prepared in the laboratory (Dacie, 1956). They should deliver 6·5 to 7 μl. It is recommended to make the capillary part from thick-walled glass so as to obtain a fair length. The pipettes are washed out with the buffer between applications.

APPARATUS

A horizontal tank suitable for cellulose acetate electrophoresis is required. All our separations were performed on a Shandon universal (Kohn) tank. The bridge gap, *i.e.*, the distance between the shoulder pieces, is set at 10 cm. With a 12 × 5 cm. strip an overlap of 1 cm. is thus obtained.

CURRENT

Current is supplied by a constant voltage power pack capable of delivering 200 v. With the buffer recommended a current of 2-2·5 m.amp. per strip would be obtained. Due to heat generation the current tends to rise during the run.

HISTIDINE ADMINISTRATION AND COLLECTION OF URINE SAMPLE

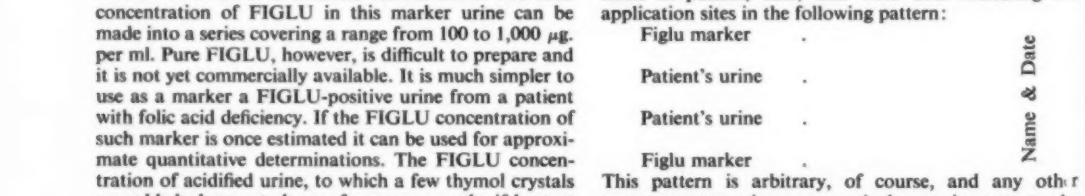
L-Histidine monohydrochloride, 15 g., is given by mouth to the patient in a fasting state and food is withheld until one hour after the administration of the dose. As the histidine is only slightly soluble it is administered by mixing it with water in a glass and then the residue in the glass is washed down with water until all the histidine has been taken.

Three hours after taking the dose the patient voids urine and this is discarded. All urine passed over the next five hours (three to eight hours after histidine) is collected in a bottle to which has been added 1 ml. of concentrated hydrochloric acid and a few thymol crystals. The urine volume is measured and an aliquot taken for analysis. The collection period of between three and eight hours is used as the determination of hourly FIGLU excretion in patients has shown the peak excretion of FIGLU to occur during this time.

TECHNIQUE OF ASSAY

The technical electrophoretic details are essentially the same as recommended for cellulose acetate electrophoresis in general (Kohn, 1959, 1960).

1 Mark the cellulose acetate strips with a soft pencil or preferably with non-diffusable ink with the name of patient, date, and with dots indicating the application sites in the following pattern:



This pattern is arbitrary, of course, and any other arrangement meeting some particular requirements can be adopted.

2 Impregnate the strip with the buffer solution. It is essential to float the strip first and to submerge only after it has soaked up from underneath.

3 After impregnation, blot the strip lightly on both sides between two sheets of filter paper. No excess moisture should be seen.

4 Place the strip in position in the apparatus.

5 Apply 6·5 to 7 μl. samples of patient's urine to the two previously marked centre spots and the FIGLU marker urine on the lateral spots. This is best performed by means of a glass capillary automatic pipette.

6 Close the lid and apply a constant voltage of 200 v. for 30 minutes. The timing may vary somewhat, depending upon conditions. The optimal time will soon be found by experience.

7 After the run is completed, remove the strips and dry them in an oven at 80 to 100°C. for 10 to 15 minutes until they are completely dry. An oven with a fan would be preferable.

8 Cut the strip into two identical halves lengthwise. Each cut part will now be a mirror image of the other.

9 Place one half strip into a container with ammonia (strip hung from the lid and a beaker with ammonia in the bottom of the container) for about 30 minutes.

10 After removing the strip from the ammonia atmosphere, dry it again at 90 to 100°C. for a few minutes, in order to drive off the ammonia.

11 STAINING Place the ninhydrin solution in a small evaporating basin or Petri dish and pass the strip slowly through the solution. It will start drying almost immediately and, at this stage, tends to curl slightly. Place it, therefore, flat between two sheets of filter paper lying between two pieces of cardboard; clip the cardboard pieces together with two Bulldog clips and put it back into the oven at 90 to 100°C. for about five minutes. The strips can then be inspected, but the colour does not develop fully until about 30 minutes. Inspect the strip by transmitted light using a bright lamp.

12 EVALUATION It has to be remembered that the whole procedure is essentially based on the comparison between the ammoniated and the non-ammoniated strips. The patterns obtained are shown in Figs. 1 and 2. Three spots can be seen on each strip; the spot nearest the cathode corresponds mainly to histidine, the one near the application line in the centre contains glycine, and the spot towards the anode is glutamic acid, which in most urines is usually very faint. If FIGLU is present in the urine it can be detected only on the ammonia-exposed strip as a spot corresponding to the mobility of the FIGLU marker and merging with the glutamic acid spot. A semi-quantitative estimation of the amount of FIGLU present can be obtained by comparing the intensity of the colour of the marker spot with the patient's FIGLU spot. If the FIGLU spot is very intense the patient's urine should be diluted in such a manner that the resulting colour intensity will be close to that of the marker urine. The dilutions should be made with acidified urine (preferably the patient's own urine before histidine was given or with any normal urine). Diluting with water is not recommended. An approximate estimation can also be performed by interpolation between two known standards.

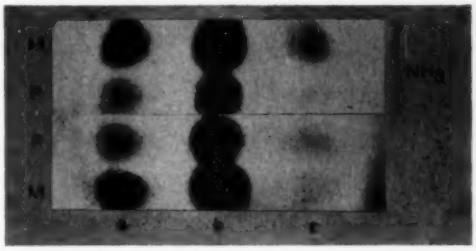


FIG. 1. *Electrophoretic pattern of a FIGLU-negative urine.*
M = marker urine containing 100 μg. FIGLU/ml. P = patient's urine.
a, histidine, b, glycine, c, glutamic acid (very faint) and FIGLU (in marker urine on ammoniated strip) 200 v., 30 min. run, contact print.
Note traces of glutamic acid on non-ammoniated strip.

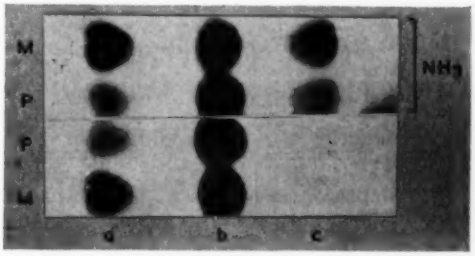


FIG. 2. *Electrophoretic pattern of a FIGLU-positive urine.*
M = marker urine containing 200 μg. of FIGLU/ml.
P = patient's urine.
a, histidine, b, glycine, c, glutamic acid and FIGLU. 200 v., 30 min. run, contact print.
Practically no glutamic acid present.

With careful technique and some experience the presence of as little as 20 μg. of FIGLU per ml. of urine can be detected. Pure FIGLU can be detected in even smaller concentrations.

The amount of FIGLU in the urine is then expressed in milligrams per hour over the five-hour collection period (between the third and the eighth hour after the administration of 15 g. histidine).

SEPARATION AT 400 V.

This is an alternative procedure which permits a clear separation of the FIGLU and the glutamic acid spots (Fig. 3). The separation can be achieved by the application of a higher voltage, *i.e.*, 400 up to 500 v. The time of running is 20 minutes. Under these conditions the basic amino-acids (histidine group) run off the strip into the cathode buffer. The sample should be applied as an

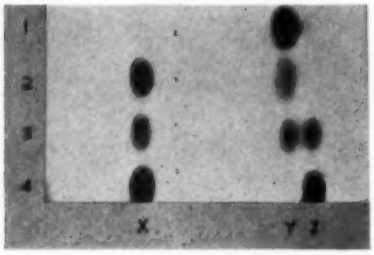


FIG. 3. *Electrophoretic pattern of a FIGLU-positive urine together with artificial mixtures of amino-acids and FIGLU showing clear separation of FIGLU from glutamic acid. 1, FIGLU; 2, FIGLU-positive urine (only trace of glutamic acid present); 3, mixture of histidine, glycine, and glutamic acid, and FIGLU; 4, mixture of histidine, glycine, and glutamic acid but without FIGLU.*
X, glycine, Y, FIGLU, Z, glutamic acid. Note absence of histidine spot which has been allowed to run off the strip into buffer. Contact print.

elliptical drop, moving the tip of the pipette slightly at right angles to the direction of the run. The current should not exceed 4-5 m.amp. if overheating is to be avoided. A rapid fall in the amperage indicates overheating and in this case semi-transparent areas can be observed. In all other respects the technique is exactly the same as with 200 v.

It should be pointed out, however, that the conditions of this method are somewhat more critical. The technique requires more experience and a power pack capable of delivering up to 500 v. The danger of overheating the strip is greater, particularly with a higher ambient temperature. Sometimes a syphoning effect, probably due to the excessive evaporation in the centre of the strip, may cause small areas or bands of discoloration after ninhydrin staining. With some experience, however, there is no difficulty in the interpretation of the results.

INVESTIGATIONS AND DIAGNOSTIC CRITERIA

All of the FIGLU determinations were carried out by one of us to whom the urine samples were sent without any clinical information on the patients. Therefore, any possible element of bias in the determination was eliminated.

Each of the patients tested had a complete blood count, and a serum vitamin B_{12} level was measured by microbiological assay with *Euglena gracilis* (Hutner, Bach, and Ross, 1956) and a serum folic acid level with the modified *L. casei* method (Waters and Mollin, 1961). A bone marrow biopsy was performed on all of the anaemic patients, and when indicated vitamin B_{12} and folic acid absorption studies were done. In most of the patients with a megaloblastic anaemia, the haematological response to 0·4 mg. folic acid or 2 μg. vitamin B_{12} a day was followed.

Patients diagnosed as idiopathic steatorrhoea had evidence of malabsorption with a typically abnormal jejunal biopsy. Patients were considered to have a nutritional folic acid deficiency if there was a megaloblastic anaemia with a normal serum B_{12} level, a poor dietary history, a normal jejunal biopsy, no evidence of malabsorption of B_{12} or folic acid, and if they had not been taking anticonvulsant drugs. Pernicious anaemia was diagnosed by the usual criteria and confirmed with radioactive B_{12} absorption tests. Patients designated as having a post-gastrectomy megaloblastic anaemia due to B_{12} deficiency had a partial or total gastrectomy, a megaloblastic bone marrow, a low serum B_{12} level, and radioactive B_{12} absorption studies similar to those of pernicious anaemia.

RESULTS

One hundred and sixty-six tests have now been carried out on 137 patients with various disease states. In Table I are summarized the results on the normal controls and patients with idiopathic steatorrhoea, nutritional folic acid deficiency, vitamin B_{12} deficiency, iron deficiency, and a few miscellaneous conditions.

Most normals showed no detectable FIGLU in the urine by this test. In an occasional normal subject a trace of FIGLU may be present but the calculated excretion has never been greater than 2 mg./hour.

In all patients with clinical evidence of folic acid deficiency the excretion has been greater than 3·5 mg./hour.

The highest levels of FIGLU with a range of 30 to 80 mg./hour were most consistently obtained in the untreated cases of idiopathic steatorrhoea, all of which showed haematological evidence of folic acid deficiency. Three patients who were well controlled on gluten-free diets for periods of one to two years showed a lower but still elevated urinary FIGLU excretion with a range of 4 to 8 mg./hour.

Several patients with idiopathic steatorrhoea were treated with intramuscular injections of 0·4 mg. of folic acid a day, and in spite of an excellent haematological response serial FIGLU determinations showed only a gradual return towards normal. In one patient, for example, the FIGLU excretion after 10 days of treatment with 0·4 mg. of folic acid a day had decreased from 44 mg./hour to 15 mg./hour. A large dose of folic acid, 10 mg. per day, was then instituted, and the FIGLU excretion became normal within another seven days.

TABLE I

URINARY FIGLU EXCRETION IN NORMAL CONTROLS AND VARIOUS DISEASE STATES

Diagnosis	*Total*	*Positive FIGLU*	*Negative FIGLU*	*FIGLU (mg./hr.) (normal 2mg./hr.)*	*Serum Folic Acid (μg./ml.) (normal >5 μg./ml.)*	*Serum Vitamin B_{12} (μμg./ml.) (normal 140–900 μμg./ml.)*
Normal controls	14	0	14	<2 *mg./hr.*	5–18	230–790
Idiopathic steatorrhea	12	12	0			
(a) Untreated	9	9	0	30–80	1·2–3·5	190–275
(b) Gluten-free diet	3	3	0	4–8	2·8–5	190–530
Nutritional folic acid deficiency	6	6	0	4–20	<1–3·5	165–390
Pernicious anaemia	7	3	4	11–48	10–23	35–80
Post-gastrectomy megaloblastic anaemia	6	1	5	6	5–20	45–120
Terminal ileum resection	1	0	1	<2 mg./hr.	17	30
Iron-deficiency anaemia	8	0	8	<2 m.g/hr.	7·4–20	240–625
Acute leukaemia on amethopterin therapy	3	3	0	24–55	0–6	570
Megaloblastic anaemia associated with anticonvulsant therapy	1	1	0	16	<1	165
Megaloblastic anaemia associated with clinical scurvy	1	1	0	12	1·7	250

The six cases of nutritional folic acid deficiency also had elevated urinary FIGLU levels but of lower magnitude, ranging from 4 to 20 mg./hour. In all of these patients with idiopathic steatorrhoea and nutritional folic acid deficiency the serum folic acid level was low.

Of the 14 cases of vitamin B_{12} deficiency (seven with pernicious anaemia, six with post-gastrectomy megaloblastic anaemia, one with a terminal ileum resection), 10 showed no increase in urinary FIGLU. However, four patients, three with pernicious anaemia and serum B_{12} levels between 40 and 65 μμg./ml. and the other with a post-gastrectomy megaloblastic anaemia and a serum B_{12} level of 45 μμg./ml., had increased FIGLU in the urine. In all 14 of these cases with vitamin B_{12} deficiency the serum folic acid level was normal. One of the patients with untreated pernicious anaemia was given 0·4 mg. of folic acid daily for 14 days without a significant haematological response.

The urinary FIGLU was not increased in eight patients with uncomplicated iron-deficiency anaemia.

In addition, increased urinary FIGLU was detected in three patients with acute leukaemia on amethopterin therapy, one case of megaloblastic anaemia related to anticonvulsant therapy, and in one patient with a megaloblastic anaemia in association with clinical scurvy. Increased FIGLU in the urine has also been present in a number of cases of widely disseminated carcinoma, haemolytic anaemia, and active liver disease. Six of seven patients with sarcoidosis also showed raised urinary FIGLU levels.

These results will be reported and discussed in greater detail elsewhere.

DISCUSSION

This method of FIGLU determination using conventional voltage electrophoresis on cellulose acetate strips is simple and practical. Our experience suggests that it is sufficiently sensitive for clinical and diagnostic purposes.

In regard to the application of the urinary excretion of FIGLU to the diagnosis of folic acid deficiency, experimental work has shown that animals made folic acid-deficient excrete increased amounts of FIGLU in the urine (Bakerman *et al.*, 1951; Silverman *et al.*, 1952; Tabor *et al.*, 1953). The majority of workers have reported a good correlation of urinary FIGLU excretion with clinical folic acid deficiency (Broquist and Luhby, 1959; Luhby *et al.*, 1959a and 1959b; Spray and Witts, 1959; Knowles *et al.*, 1960). However, others have claimed that the test is not uniformly reliable. These reports, not as yet published in detail, indicate that some patients with pernicious anaemia, responding completely to vitamin B_{12} alone, have significant amounts of FIGLU in their urine and some patients with folic acid deficiency do not have FIGLU in their urine (Rucknagel, La Du, Laster, Seegmiller, Daft, Silverman, and Pitney; Marshall, Jandl, Castle, and Hiatt; Herbert, Baker, Frank, Pasher, Sobotka, and Wasserman; as cited in Herbert, Baker, Frank, Pasher, Sobotka and Wasserman, 1960).

In our experience so far, all patients with clinical and microbiological evidence of folic acid deficiency, have had an increased urinary excretion of FIGLU (Table I). However, four of 14 patients with vitamin B_{12} deficiency have also shown an increased urinary FIGLU excretion. The explanation for this finding

is uncertain but it may possibly represent concomitant or secondary folic acid deficiency (Chanarin, Mollin, and Anderson, 1958). Nevertheless, the serum folic acid level was normal in all four of these cases as well as in the other 10 cases of vitamin B_{12} deficiency (Table I). In all four cases the excessive excretion of FIGLU disappeared when the patients were treated with vitamin B_{12}. It is therefore possible that the excessive excretion of FIGLU by these patients was due to a defect in folic acid metabolism caused by the deficiency of vitamin B_{12}.

A number of patients with disseminated carcinoma, active liver disease, and sarcoidosis, many of whom showed no megaloblastic change in the marrow, had an elevated urinary excretion of FIGLU. Whether this represents tissue depletion of folic acid or whether it is due to deficiency of FIGLU transferase or folic acid-reducing enzymes, as suggested in alcoholic cirrhosis of the liver (Carter, Schaffner, and Heller, 1960), is not known. In this connexion the persistence of the increased FIGLU excretion in patients with folic acid deficiency while on treatment with 0·4 mg. of folic acid daily, in spite of an excellent haematological response, suggests that the test may be very sensitive to depletion of folic acid in the tissues. However, until more is known about the specificity and sensitivity of FIGLU excretion, the meaning of these findings is uncertain.

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The urinary excretion of assayable vitamin B_{12} and radioactivity after parenteral ^{58}Co B_{12} in man

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SYNOPSIS Evidence is presented that after injection of radioactive vitamin B_{12} in man, there is a close correlation between the amount of radioactivity excreted and the amount of assayable vitamin B_{12} excreted, and thus that the amount of radioactivity excreted is a true measure of the vitamin B_{12} excreted. The possible reasons for this occurrence are discussed and it is suggested that in the body vitamin B_{12} does not exist as such but as an analogue or active derivative.

It is generally assumed that, in man, vitamin B_{12} (cyanocobalamin) is absorbed from the intestinal tract and functions in the body as an active substance without undergoing any chemical change. This view is based largely on the results of recovery experiments in which known amounts of vitamin B_{12} are added to tissues; the increase in microbiological activity, as measured by assay methods considered to be specific for vitamin B_{12}, is proportional to the amount of added vitamin B_{12} and hence it seems likely that the original microbiological activity of the tissue is also due to vitamin B_{12}.

If a patient who had no vitamin B_{12} in his body and was not excreting any radioactive substance in his urine were given an injection of radioactive vitamin B_{12} it would therefore be a reasonable assumption that the amount of radioactivity then found in the urine would be a true indication of the amount excreted via the kidney. For example, if this hypothetical patient were given 1,000 μg. radioactive vitamin B_{12} intramuscularly and 20% of the radioactivity was found in the urine then that 20% would represent 200 μg. of vitamin B_{12}. The normal subject, however, has between 1,500 and 5,000 μg. of non-radioactive vitamin B_{12} in his body and the simple process outlined above cannot be assumed to occur because the possibility must be considered that the injected radioactive B_{12} might equilibrate or mix with the non-radioactive body stores. Under these circumstances the amount of radioactivity appearing in the urine after an injection of radioactive vitamin B_{12} would not be a true indication of the amount excreted but would represent only a fraction of that excreted. For example, if a patient with 3,000 μg. non-radioactive vitamin B_{12} in his body were given 1,000 μg. radioactive vitamin B_{12} intramuscularly and 20% of the radioactivity was excreted in the urine then that 20% would represent 20% of (1,000 + a proportion of 3,000) microgrammes vitamin B_{12} depending on the completeness of equilibration.

Preliminary investigations showed that the normal subject excreted about 70% of the radioactivity of an injection of 1,000 μg. radioactive B_{12}: it was obvious that complete equilibration could not have taken place otherwise this would have represented about 2,800 μg. if the body stores were 3,000 μg. It was clearly important to determine whether any significant degree of equilibration did in fact occur and this could be detected by injecting radioactive vitamin B_{12} and comparing the amount excreted in the urine as measured by the radioactivity present and by microbiological assay. If there was agreement with the two methods this would indicate that equilibration had not taken place whereas if equilibration did occur the amount of assayable vitamin B_{12} in the urine would be greatly in excess of the amount indicated by the radioactivity present. For example, if a subject were given 1,000 μg. radioactive vitamin B_{12} and 50% of the radioactivity were excreted and a total of 500 μg. of vitamin B_{12} was found by assay then this would be evidence against equilibration of significant degree: if, however, 50% of the radioactivity were excreted and 750 μg. of vitamin B_{12} were found by assay this would suggest that the 1,000 μg. of radioactive vitamin B_{12} had equilibrated with 500 μg. of the body stores of non-radioactive vitamin B_{12}.

Such an investigation supposes that the assay is accurate and fails to take into account the possibility of a constant error which would cause misleading

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results. This error can be detected by duplicating the experiment in patients who have no body stores of vitamin B_{12}. For example, if a normal subject were given 1,000 μg. radioactive vitamin B_{12} intramuscularly and 50% of the radioactivity were excreted in the urine and 600 μg of vitamin B_{12} was found by assay this would suggest either that there had been some equilibration or that the assay result was too high due to a constant error. If the same results were obtained in a patient with no body stores of vitamin B_{12} then it would be clear evidence that the assay was giving a high result. Repeated injections of radioactive vitamin B_{12} can be given to such control patients without prejudicing the results. If equilibration did occur then the injected radioactive vitamin B_{12} would equilibrate with that remaining in the body from the previous injection. The use of control subjects would not only serve to reveal a constant error in the assay if such were present but would also reveal the degree of any equilibration which took place.

If it could be shown that equilibration of injected radioactive vitamin B_{12} and non-radioactive body stores of vitamin B_{12} did not occur then this finding would be of both practical and academic importance. Such a conclusion is drawn from the studies reported here.

PLAN OF THE EXPERIMENT

The object of the experiments was to compare the urinary excretion of radioactivity and of total assayable vitamin B_{12} after parenteral injection of ^{58}Co vitamin B_{12} in normal subjects and control subjects who were suffering from vitamin B_{12} deficiency. To cover the usual range of doses used in clinical practical practice three different doses of ^{58}Co vitamin B_{12} were used.

PATIENTS, MATERIALS, AND METHODS

The control patients were seven in-patients suffering from severe vitamin B_{12} deficiency. Five were diagnosed as having Addisonian pernicious anaemia on the basis of a macrocytic anaemia, megaloblastic bone marrow, histamine-fast achlorhydria, low serum vitamin B_{12} level, and a reticulocyte response and rise in peripheral blood values after treatment with parenteral ^{58}Co vitamin B_{12} only; after the study reported here the absorption of oral ^{58}Co vitamin B_{12} was studied in these patients with and without intrinsic factor by the method of Schilling (1953) and the results supported the diagnosis. One patient was diagnosed as having adult coeliac disease on the basis of a macrocytic anaemia, megaloblastic bone marrow, free gastric acid, excess fat in the faeces, a low serum vitamin B_{12} level, and reticulocyte response and rise in peripheral blood values after treatment with ^{58}Co vitamin B_{12} only; Schilling tests on this patient showed malabsorption of vitamin B_{12} unaffected by the addition of intrinsic factor. One patient came under observation with a macrocytic anaemia, megaloblastic bone marrow, and a low serum vitamin B_{12} level four years after total gastrectomy. He also responded to parenteral ^{58}Co vitamin B_{12} and Schilling tests showed that he failed to absorb it unless it was given with intrinsic factor. The control patients were treated by ^{58}Co vitamin B_{12} intramuscularly daily.

The normal patients were in-patients with a variety of diseases in which depletion of body vitamin B_{12} stores is not a known occurrence. All had normal serum vitamin B_{12} levels. These patients were given a single intramuscular injection of ^{58}Co vitamin B_{12}.

The nature and objects of the experiment were explained to the patients, all of whom cooperated willingly.

^{58}Co vitamin B_{12} was obtained from the Radiochemical Centre, Amersham, and diluted to a concentration of 0·5 μg., specific activity 0·5 μc., in 5 ml. normal saline, dispensed in dark glass rubber-capped bottles, autoclaved and stored at +4°C when not in use. The vitamin B_{12} content was checked by microbiological assay every week the batch was in use. Ampoules of ^{58}Co vitamin B_{12} for injection were prepared in the three dose ranges by adding 2 ml. of the ^{58}Co vitamin B_{12} solution to 1,140 and 540 μg. non-radioactive vitamin B_{12} and 3 ml. of the ^{58}Co vitamin B_{12} solution to 54 μg. non-radioactive vitamin B_{12}. The same tuberculin syringe was used for the preparation of these solutions and the preparation of the standard for isotope counting. Intramuscular injections were made by standard methods.

Urine was collected directly into acid-washed dark glass bottles. Pretreatment 24-hour collections were made on all patients. The total volume was noted and aliquots taken for isotope counting and microbiological assay. Samples for assay were diluted as required with vitamin B_{12}-free water and stored at −20°C. till used: assays were performed as soon as possible.

ISOTOPIC METHODS The radioactivity in 450 ml. aliquots of urine contained in plastic bottles was measured by the end-on method in a suitably modified Ecko scintillation counter type N 550 with thallium-activated sodium iodide crystal, 3·81 cm. diameter and 2·54 cm. deep, shielded by 3 in. of lead, and an Ecko automatic scaler type N 530A. A standard containing 0·2 μc. ^{58}Co vitamin B_{12} and 2,000 μg. vitamin B_{12} as a carrier in 450 ml. water was used. All samples were counted until 10,000 counts had been recorded in the 1,140 μg. and 540 μg. series, or until 5,000 counts had been recorded in the 54 μg. series.

MICROBIOLOGICAL ASSAYS were conducted by a modification of the methods of Ross (1952) and Hutner, Bach, and Ross (1956) using *Euglena gracilis* 3 strain as the test organism. Samples were assayed in at least two different assay batches.

STATISTICAL METHODS The results were analysed by standard statistical methods.

RESULTS

No radioactivity and only a negligible amount of assayable vitamin B_{12} was found in the pretreatment

collections in normal and in control patients. The results of the microbiological assays expressed as total assayable vitamin B_{12} excreted in micrograms and the amount of radioactivity excreted expressed as a percentage of the dose together with the regression line and 95% confidence limits for the control patients are plotted in Figs. 1, 2, and 3. The essential statistical results are set out in Table I.

It will be observed that the mean results for excretion in the control and normal patients do not differ greatly. This aspect will be discussed in a further communication.

DISCUSSION

A clear implication of the results is that, for practical purposes, parenteral radioactive vitamin B_{12}, in the doses used, does not equilibrate with the body stores of non-radioactive vitamin B_{12}, at least in the 24 hours after injection. In the 54 μg. series it is permissible for the regression lines and standard devia-

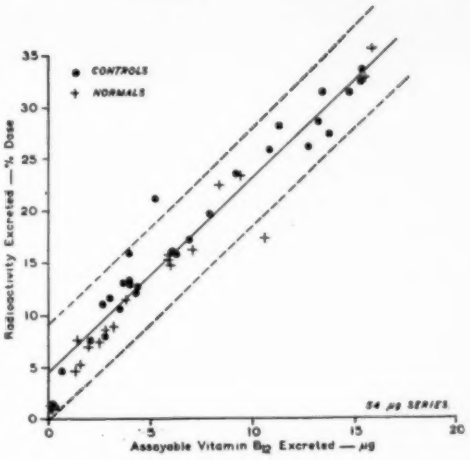

FIG. 1. *Correlation of assayable vitamin B_{12} and radioactivity excreted after injections of 54 μg. ^{58}Co vitamin B_{12} in normal and control subjects.*

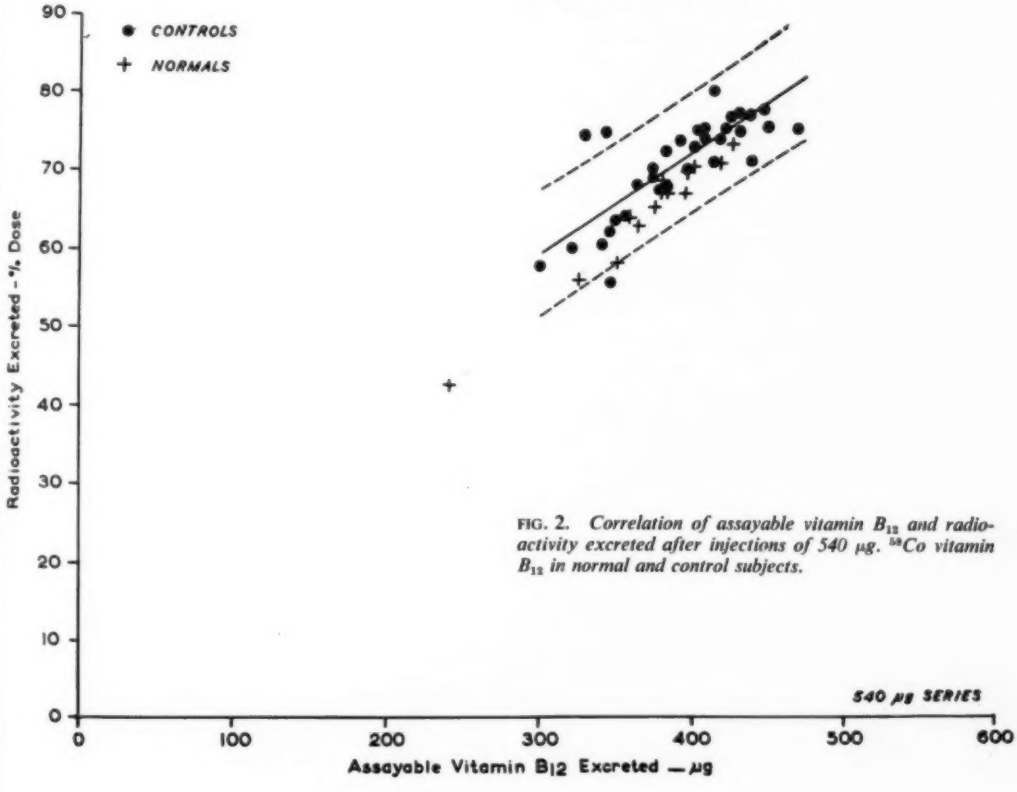

FIG. 2. *Correlation of assayable vitamin B_{12} and radioactivity excreted after injections of 540 μg. ^{58}Co vitamin B_{12} in normal and control subjects.*

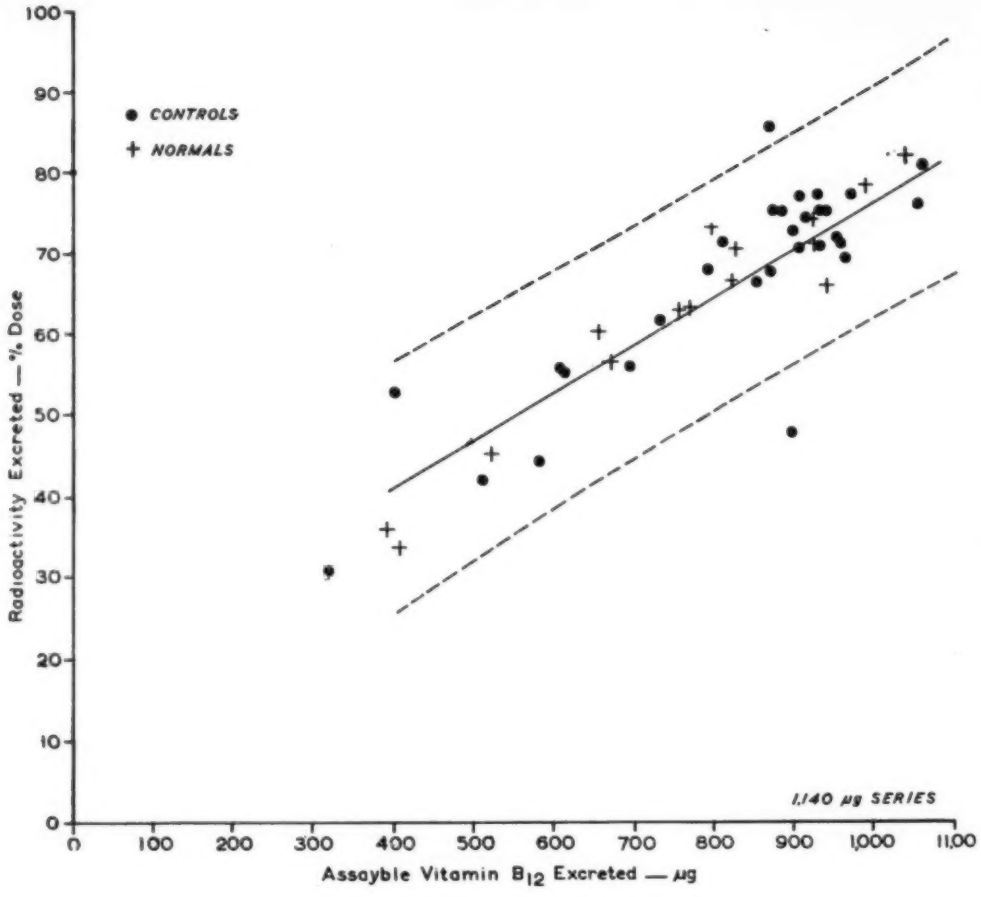

FIG. 3. *Correlation of assayable vitamin B_{12} and radioactivity excreted after injections of 1,140 μg. ^{58}Co vitamin B_{12} in normal and control subjects.*

TABLE I

ESSENTIAL STATISTICAL RESULTS OBTAINED IN THE INVESTIGATION

Series (μg.)	*Patients*	*No. of Readings*	*Mean Values*		*Coefficient of Correlation (r)*	*Regression of Isotope (x) on Assay (y)*	*S.E. of Estimate (%)*
			Assayable B_{12} (μg.)	*Radioactivity % of Dose*			
54	Controls	30	6·83	17·06	0·9740	x = 1·8809y + 4·2131	2·256
54	Normals	18	5·828	14·12	0·9546	x = 1·9374y + 2·8259	2·078
54	Controls	33	392·36	71·27	0·8220	x = 0·12731y + 21·314	3·70
540	Normals	14	373·00	64·72	0·9482	x = 0·15675y + 6·2541	1·88
1,140	Controls	30	825·97	66·63	0·8517	x = 0·05937y + 17·595	6·911
1,140	Normals	15	827·88	69·97	0·8270	x = 0·056961y + 22·807	5·079

tion curves to be extended to meet the abscissa and ordinate. The lower SD curve for the control group (Fig. 1) lies on the point abscissa O, ordinate O, while for the normal group the lower SD curve meets the ordinate at the point abscissa O ordinate −1·88, the regression line for the controls meets the ordinate at the point abscissa O ordinate 2·83 and the upper SD curve for the controls meets the ordinate at the point abscissa O ordinate 7·54. These findings are good evidence that the lack of equilibration occurs in the entire dose range up to 1,140 μg.

It is difficult to explain the phenomenon on the basis of the concept that vitamin B_{12} is present as such in man. An obvious explanation would be that the injected radioactive vitamin B_{12} is excreted so rapidly that there is insufficient time for equilibration to occur; detailed observations on the rate of excretion of injected ^{58}Co vitamin B_{12} suggest that this explanation is unlikely to be wholly satisfactory.

The explanation may be related to the fact that the preponderant mass of vitamin B_{12} in the body is tissue bound. The process of binding involves at least physical changes, since the assayable vitamin B_{12} in liver is microbiologically active but is not dialysable, and it is pertinent to consider whether the process may not also involve chemical changes in the structure of the vitamin B_{12} molecule, *i.e.*, whether vitamin B_{12} absorbed from dietary sources is converted to an analogue in the process of binding to body tissues. If this were the case one would not expect the injected radioactive vitamin B_{12} to equilibrate with the analogue until it also has been converted by the process of binding, by which time it would be fixed to the tissues and no longer free to be excreted. The proportion which is excreted would be that which the tissues have been unable to bind in the time available.

Recently biochemical evidence has been produced which suggests that a very considerable proportion of the cobalamins in rabbit liver are present not as vitamin B_{12} but as coenzymes which may be the metabolically active form of vitamin B_{12}. There is also evidence that these coenzymes can be converted to vitamin B_{12} by procedures commonly used in microbiological assays of tissues such as exposure to heat, light, or to cyanide ions (Weissbach, Toohey, and Barker, 1959). This suggests that in microbiological assays the substance which is actually measured is vitamin B_{12} derived from body stores of the microbiologically active form. In addition, one of these coenzymes, 5, 6-dimethylbenzimidazolylcobamide coenzyme, has been shown to be as metabolically active in patients with pernicious anaemia as vitamin B_{12} at the same dosage level (Wasserman, Estren, Brody, and Herbert, 1960).

It is also clear from the results that when man is given such injections of radioactive vitamin B_{12} the radioactivity found in the urine in the subsequent 24 hours is a true indication of the amount of assayable vitamin B_{12} excreted and that this occurs regardless of its body stores. This finding is of considerable practical importance for it means that the isotope method can be used with confidence instead of the microbiological assay in determining the total urinary loss of vitamin B_{12} after an injection of radioactive vitamin B_{12}, at least for the doses used. The isotope method, which makes use of a very small dose of radioactivity, has obvious advantages over the assay method; as a technical method it is inherently more accurate and reliable results may be obtained quickly and with little expenditure of time or labour. The practical application of this method will be dealt with in subsequent papers.

I am grateful to Dr. J. A. W. McCluskie for his advice and criticism and to Professor E. J. Wayne for facilities in his department. It is a pleasure to thank Dr. R. A. Robb for his advice on the statistical problems and Dr. M. M. Bluhm for his advice on the isotopic aspects. The ^{58}Co vitamin B_{12} was supplied by the Regional Physics Department, Western Regional Hospital Board.

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A suggested schedule for the rapid investigation of acute haemostatic failure

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SYNOPSIS A small group of tests is proposed for the rapid investigation of acute bleeding states. These techniques are within the compass of many clinical laboratories, and the whole scheme may be run through in one to one-and-a-half hours.

Acute bleeding states may arise in association with concealed accidental haemorrhage and other obstetrical complications, surgery of the lung, carcinomatosis, and other conditions. It is characteristic for multiple haemostatic defects to be produced, the pattern varying from case to case. If the haematologist is to contribute to clinical management he must be able quickly to identify the cause of bleeding so that he may be able to advise promptly on the appropriate replacement therapy or on the risks of surgery.

The following scheme of investigation is therefore offered for a rapid appraisal of those facets of haemostasis which usually seem to be affected. A fuller discussion has been given by Ingram, Norris, and Tanner (1960) with an illustrative obstetrical case history; an example from chest surgery has been described by Ingram and Mann (1959).

The two commonest processes underlying acute failures of haemostasis appear to be defibrination and fibrinolysis; it has been thought that either may be initiated by an escape of tissue material into the blood stream. More rarely, a heparin-like inhibitor may appear in the blood.

Defibrination and fibrinolysis may occur separately or together. Their effects are similar, for they both characteristically produce thrombocytopenia, fibrinogenopenia, and deficiencies of antihaemophilic globulin (AHG) and factor V. The following scheme is therefore designed to test these four components, as well as to detect fibrinolysis and heparinaemia. The other types of inhibitors which have been described do not appear to develop so acutely (with the possible exceptions of one associated with fibrinolysis described by Niewiarowski, Kowalski, and Stachurska, 1959, and of the effect discussed by von Kaulla and Swan, 1958).

A few notes on treatment are appended.

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METHODS

All tests except the platelet count are made on citrated plasma (9 volumes of blood added to 1 volume 3·8% sodium citrate). The following five tests are used: (1) Platelet count; (2) Quick's prothrombin time for activity of factor V (a long prothrombin time due to factor V deficiency would be shortened by the addition of $Al(OH)_3$-treated normal plasma 1 in 5 or 1 in 10); (3) rapid fibrinogen determination; (4) a simple modification of the thromboplastin generation screening test of Hicks and Pitney (1957) for antihaemophilic globulin activity (although also affected by factor V); (5) the thrombin clotting time, providing a fibrinogenolysis test (adapted from Wilhelm, Miles, and MacKay, 1955), and a simple test for heparinaemia. Tests (1) and (2) are, of course, done by the usual methods. Tests (3), (4), and (5) are described below.

TEST 3 This is the determination of the weight of fibrin obtained on clotting a measured volume of plasma (Ingram, 1952a), omitting drying the clot in hot air.

Method Equal volumes of plasma and M/40 CaCl$_2$ (say 2·0 ml.) are mixed and incubated for about 10 minutes, a wooden applicator stick being placed in the tube. When a firm clot has formed this is wound on to the stick, expressing the serum. The stick is then transferred to a tube of water and the serum returned to incubate in case further clot forms, which is harvested as before. The total clot is then peeled off the stick (like stripping the rubber grip off the handle of a cricket bat) and pressed between filter papers, and then transferred to acetone, to dry and harden, for exactly 10 minutes. After decanting the clot is transferred to the warm hand, and the remaining acetone rapidly evaporated by gentle blowing; the clot is then immediately weighed to the nearest 0·2 mg.

The clot may of course be determined by other methods if preferred, but gravimetry has been found very convenient for a 24-hour service, since a chemical balance requires no 'warming up' and most laboratory workers are familiar with its use.

$$\frac{\text{Weight of clot}}{\text{Volume of plasma}} = \text{concentration of fibrinogen in citrated plasma}$$

$$e.g., \frac{7{\cdot}3 \text{ mg.}}{2{\cdot}1 \text{ ml.}} = 0{\cdot}35 \text{ g./100 ml. (the conventional unit)}$$

Errors The true value will be overestimated by the water remaining in the clot, and underestimated by the dilution of the plasma with the citrate. These errors may be corrected as follows: (*a*) For retained water, reduce the observed weight of the clot by subtracting 15% plus an additional ½% per mg. observed weight. (This correction was determined empirically by observing the further loss in weight which occurred after heating clots in hot air as described in the original method.) (*b*) For citrate dilution, multiply the value of the concentration of fibrinogen (as amended by (*a*) above) per millilitre of plasma by

$$\frac{\text{Percentage plasma in citrated blood}}{\text{Percentage plasma minus percentage citrate}}$$

(Since the proportion of citrate in the whole citrated blood is known, the correction may be calculated from the haematocrit of this blood.) Note that these corrections tend to cancel each other, and that in fibrinogenopenia, when a very small clot is obtained, they have a small effect anyway and so for clinical purposes they may often be ignored.

The following is an example when the haematocrit of the citrated blood is 34%

$100 - 34 = 66$

½% × 7·3 = 4%; 15% + 4% = 19%

19% of 7·3 = 1·4 mg.; 7·3 − 1·4 = 5·9 mg.

$$\text{corrected value} = \frac{5{\cdot}9 \text{ mg.}}{2{\cdot}2 \text{ ml.}} \times \frac{66}{66-10} = 0{\cdot}32 \text{ g./100 ml.}$$

To estimate a patient's plasma fibrinogen deficit, reckon the blood volume as 1/10 body weight; calculate the plasma volume from this estimate and the venous haematocrit; multiply the value obtained by the observed fibrinogen concentration and this gives an estimate of the total plasma fibrinogen. Similarly calculate the total plasma fibrinogen had the concentration been normal (say 0·3 g./100 ml.). The difference between these two calculated values estimates the plasma fibrinogen deficit.

TEST 4 Hicks and Pitney's (1957) test may be reduced to its simplest terms by adopting the 'running' design suggested by Biggs, Eveling, and Richards (1955). The method is described here in detail and outlined in Table I.

Method Mix 0·1 ml. of each of a platelet substitute (see below), control plasma 1 in 10, and M/40 CaCl, and put to incubate with a pipette standing in the tube. One minute later mix 0·1 ml. platelet substitute, 0·1 ml. of patient's plasma 1 in 10, and 0·1 ml. of M/40 $CaCl_2$ in a second tube and put to incubate with a pipette in the tube. Again, one minute later, mix 0·1 ml. of platelet substitute, 0·05 ml. of normal plasma 1 in 10 and 0·05 ml. patient's plasma 1 in 10, and 0·1 ml. M/40 $CaCl_2$, and put to incubate, with a pipette, in a third tube. At the next minute transfer 0·1 ml. from the first tube to 0·1 ml. of normal citrated plasma to which 0·1 ml. of M/40 $CaCl_2$ has just been added (5 to 10 sec. before) and measure the clotting time. At the next minute perform this procedure on the second tube and at the next minute for the third tube. At the following three minutes repeat the procedure again for the first, second, and third tubes in that order. It is convenient to arrange the three reaction tubes and six clotting time tubes (containing normal plasma) in the water bath before starting the test. It will be apparent that this procedure yields a three-minute and a six-minute sample by the Hicks and Pitney procedure for normal plasma, the patient's plasma, and a mixture of the two so that in one run it becomes apparent whether the patient's plasma behaves abnormally in this test and if so whether this is due to a deficiency (when the results from the third tube will approximate to those of the first) or to an inhibitor (in which case the results of the third tube will approximate to those of the second). For confirmation the whole procedure may be repeated in the reverse order, *i.e.*, 50 : 50 mixture, patient's plasma, control plasma, thus obtaining two replicate readings at each sampling time from each tube. The reversal of the order represents an attempt to eliminate a trend due to the passage of time (Ingram, 1955).

TABLE I

SIMPLIFIED THROMBOPLASTIN GENERATION SCREENING TEST

Time (min.)	*Action*
0	Set up tube 1 (normal plasma)
1	Set up tube 2 (patient's plasma)
2	Set up tube 3 (50 : 50 mixture of plasmas)
3	3 min. sample from tube 1
4	3 min. sample from tube 2
5	3 min. sample from tube 3
6	6 min. sample from tube 1
7	6 min. sample from tube 2
8	6 min. sample from tube 3

Each tube is set up with 0·1 ml. of each of the appropriate plasma 1 in 10, platelet substitute, and M/40 $CaCl_2$, and at the times indicated is sampled onto 0·1 ml. of normal undiluted citrated plasma to which has just been added 0·1 ml. M/40 $CaCl_2$.

ALTERNATIVE PROCEDURE CONTROLLING 'CONTACT' The above test is conveniently rapid and adequate to detect gross defects but it ignores differences in the degree of 'contact' to which the plasmas have been subjected. Variations between the 'contacting' of the samples may lead to variations in the rate of their reactivity, and may thus blurr the differences between normal and mildly abnormal plasmas.

It is difficult to ensure that contact is equally prevented in all samples but an equal, vigorous contacting can be applied by a brief preincubation with kaolin (Margolis, 1957, 1958). This provides a better technique, although slight inconveniences introduced by this procedure are that an additional reagent is required, and that through its action, thromboplastin generation becomes so rapid that is is more informative to sample at 2 min. and 4 min. instead of at 3 min. and at 6 min.; hence, in the 'running'

design, only two, and not three, reactions can be handled concurrently. The 50 : 50 mixture of patient's and control plasma must therefore be tested separately. (The design thus allows this mixture to be paired with a test on 1 in 20 control plasma for a comparison with the effect of simple dilution if desired.) Note that by 2 min. the reaction mixture containing control plasma may well have reached maximum activity so that it may in fact be simpler, for occasional use, not to employ the running design, but to test each plasma separately in the traditional manner and to sample at 1, 2, 3, and 4 min. after completing the reaction mixtures, having preincubated the kaolin and the plasma for two minutes. A running design can then be used to decide between deficiency and an inhibitor, and to test the effects of various additions, as described immediately below.

Method A convenient stock suspension contains light kaolin, 10 mg./ml.; this is diluted 1 in 10 for use. Place 0·05 ml. of the working suspension in a tube and add 0·1 ml. of control plasma 1 in 10; mix and put to incubate. For the running design, one minute later make a corresponding mixture with patient's plasma 1 in 10 and put to incubate. At the next minute, add platelet substitute and M/40 $CaCl_2$ to the control tube, as in the previous method; and at the following minute similarly complete the reaction mixture for the patient's plasma. At the subsequent four successive minutes subsample the control reaction, the patient's reaction, and again the control reaction and the patient's reaction, according to the previous method. Each plasma will then have received two minutes' preincubation with kaolin and will have yielded samples at two and four minutes after the completion of the reaction mixtures. The principles of interpretation are of course on the same lines as in the previous method.

If the test suggests that the patient's plasma is deficient rather than inhibitory do another run in which the control plasma only has been adsorbed with alumina (Biggs and Macfarlane, 1957, pp. 387, 388) before dilution. This should of course give an abnormal run when tested alone, but if in the 50 : 50 mixture it corrects the patient's plasma about as well as did the untreated normal plasma, then the patient's blood must be deficient in either antihaemophilic factor or factor V; and if replacement infusion is indicated, fresh normal acid-citrate-dextrose (A.C.D.) plasma should be given. If adsorbed normal plasma does not correct but if, on the other hand, aged normal serum 1 in 10 corrects the patient's plasma about as well as did the untreated normal plasma in the original test then the patient may be treated with A.C.D. plasma which has been stored for up to one month at −20°C., since the 'serum' factors appear to be stable in this material (Brafield and Case, 1956).

PLATELET SUBSTITUTES Platelet suspensions are tedious to prepare and do not keep well but several substitutes are available. A preparation from brain was described by Bell and Alton (1954); a soy-bean phospholipid, Inosithin, may also be used, at about 0·02 g.%[1]

[1]Associated Concentrates, Inc., 57-01 37nd Ave., Woodside, N.Y.; obtainable in the U.K. through V. A. Howe & Co. Ltd., 46 Pembridge Road, London, W.11.

TEST 5 Fibrinolysin acts in the body as a fibrinogenolysin, and this is also the basis of the test described by Wilhelm *et al.* (1955). Separate samples of the patient's and the control plasmas, *e.g.*, 2·0 ml., are incubated concurrently, and at intervals 0·1 ml. samples are withdrawn and the thrombin clotting times measured. If the fibrinogen is being gradually destroyed in the patient's incubated sample the thrombin clotting times on this plasma will progressively lengthen.

Thrombin clotting time test Human thrombin is diluted with M/80 $CaCl_2$ so that when 0·2 ml. is added to 0·1 ml. of normal citrated plasma the clotting time is approximately 10 sec. This test is also sensitive to the presence of heparin so that if readings are obtained from the two plasmas immediately they are put to incubate and the patient's plasma gives a longer clotting time *ab initio*, this might be due to a heparin-like anticoagulant or to a pre-existing reduction in the fibrinogen concentration of the plasma. If the clotting time is prolonged by heparin, the difference between the patient's and the control times will be reduced by adding to each plasma a volume of toluidine blue solution 1 in 1,000 to 1 in 10,000 before repeating the test; if the long clotting time is due to hypofibrinogenaemia, it will similarly be shortened by adding a volume of fibrinogen solution, approximately 200 mg. per 100 ml. Heparinaemia is, of course, rare, and once this has been excluded, proceed with the fibrinogenolysis test by measuring the thrombin clotting time on further 0·1 ml. subsamples from each plasma at intervals of, say five, 10, 20, 40, and 60 minutes according to the rate of change observed. If the fibrinogen concentration is already low in the patient's sample, fibrinogen must be added to both incubated plasmas in order to work this test, but either way, if no consistent difference between the plasmas is apparent after two hours' incubation, the test is negative for clinical purposes. Note that the control clotting time may also lengthen somewhat as the test proceeds, but this is immaterial, since the significant finding is an increasing difference (conveniently expressed as a proportion) between the clotting times from the two plasmas.

SETTING UP THE TESTS It will be apparent that these tests may be run concurrently and the following plan is suggested:

(1) Obtain 10 ml. citrated blood from the patient and from a control subject: (2) set up the haematocrit of the patient's citrated blood, and also (3) the patient's platelet count, either on the venous or a capillary blood sample according to local practice. (4) Spin both the citrated bloods (say, 3,000 r.p.m. for 10 min.) to obtain citrated plasma. (5) Set up the fibrinogenolysis test and (6) take the initial readings to exclude heparinaemia; (7) set up the fibrinogen estimation and (8) take the next pair of readings from the fibrinogenolysis test; (9) do Quick's prothrombin time in the two plasmas; (10) repeat the fibrinogenolysis test; (11) examine the fibrinogen estimation tube and harvest the clot if it seems ready; (12) prepare the reagents for the Hicks and Pitney test and (13) repeat the fibroinogenolysis test if the rate of change in previous readings warrants it. It then only remains (14) to count the platelets, (15) perform the Hicks

and Pitney test, (16) weigh the clot, and (17) repeat the fibrinogenolysis test in the most convenient order. The whole procedure takes an hour to an hour and a half, single handed.

Note that the following reagents may be maintained at −20°C., from which dilutions may be prepared for immediate use: (1) Human thrombin[1], approximately 50 units per ml., and (2) human fibrinogen, 1 g. per 100 ml. It has been found that the inclusion of a quarter-volume of the veronal buffer of Owren (1949) seems to prevent denaturation on repeated freezing and thawing. (3) The platelet substitute as a colloidal solution of Inosithin, 1 g. per 100 ml., or Bell and Alton's (1954) brain extract as described by the authors.

DISCUSSION

These tests are intended for laboratory use under the supervision of the clinical pathologist and are not bed-side tests (in the sense of tests to be performed by clinicians[2]) although they are simple enough to be done in a side-room equipped with a centrifuge, water bath, and the basic glassware. There is thus no mention of tests made on whole blood, such as the clotting time or the clot observation test, which are less efficient than the tests described in this paper and are only worth carrying out when laboratory facilities are not available.

The thrombin titre test of Sharp, Howie, Biggs, and Methuen (1958) is not mentioned because it is thought that the same information will be given by the test for fibrinogenolysis described above and that this test is more convenient to work in with other procedures which are proposed.

For the detection of a deficiency of AHG in the acquired bleeding states, the two-stage test of Hicks and Pitney is preferred to one-stage tests, *e.g.*, of the type proposed by Margolis (1958), because in the circumstances under discussion here, the one-stage tests might give abnormal results due to a deficiency of fibrinogen or even because prothrombin had become seriously depleted. These tests would therefore be difficult to interpret in terms of the early stages of blood coagulation which involve AHG. In the two-stage test, only the early phases of coagulation depend on materials supplied by the patient's plasma so that interpretation should be easier.

FIBRINOGEN ESTIMATION Numerous techniques for the estimation of plasma fibrinogen concentration are of course available and other methods than that described may be preferred by various workers. It is, however, thought important to estimate what coagulates rather than what precipitates at a defined salt concentration or under other unphysiological physical conditions, and the described method has been found convenient and rapid for emergency use.

The normal ranges of clotting tests are difficult to define briefly. Normal adult values for the non-pregnant state are given or discussed by Ingram (1952b) for fibrinogen concentration, by Hicks and Pitney (1957) for the thromboplastin generation screening test, and by Ingram and Armitage (1959) for Quick's prothrombin time; published normal values for late pregnancy are discussed and a personal series is given by Ingram *et al.* (1960). In particular, the last paper gives the basis for choosing 3 min. and 6 min. sampling times in the Hicks and Pitney test.

INDICATIONS FOR TREATMENT There are two principles in treating acute haemostatic failure. Fundamentally, the patient's clinical condition must be resolved or limited, and if this can be achieved the patient's haemostatic mechanisms will return to normal. Meanwhile, as a holding operation, the haemostatic defects should be kept above their critical levels by infusing the appropriate blood derivatives (fibrinogen, plasma, etc.). It is to obtain evidence on which to base the choice of the appropriate infusion that the above tests are designed. The clearest indication for infusing a particular component found to be abnormally low in the patient's blood is to show by successive tests that the deficiency is progressively worsening, but clearly it is usually necessary in practice to decide on the results of single estimates. It may therefore be useful to suggest critical levels below which infusion should be considered. Table II gives working values for the platelet count, the fibrinogen estimation, and the thromboplastin generation screening test.

A potent and apparently safe antifibrinolytic drug is now available. Nilsson, Sjoerdsma, and Waldenström (1960) gave ϵ-amino caproic acid (ϵACA) in doses of up to 6 g. intravenously, and by mouth to a daily total of 36 g., with evidence of antifibrinolytic effect *in vivo*, to patients showing marked fibrinolysis. Where fibrinolysis has gravely depleted haemostatic factors these deficiencies should presumably also be made good as suggested above.

After treatment with blood or blood derivatives, the tests found initially abnormal may be repeated to check progress. In particular, if purified fibrinogen has been given, the fibrinogen estimation may be repeated at the end of the infusion and again after an interval of some hours. If the reading immediately after the infusion is lower than would have been

[1] Human thrombin and fibrinogen may be available for laboratory use in small quantities from time to time from the Blood Products Laboratory, The Lister Institute, Elstree, Herts (Dr. W. d'A. Maycock).

[2] A simple fibrinogen estimation for bedside use, intended as a clinical screening test, has been described in a previous paper (Ingram and Mitchell, 1960).

TABLE II

INDICATIONS BASED ON SUGGESTED CRITICAL VALUES OF PLATELET COUNT, PLASMA FIBRINOGEN CONCENTRATION, AND THROMBOPLASTIN GENERATION SCREENING TEST

Test	*Critical Value*	*Material to be Infused*	*Dose*
Platelet count	100,000/mm.[3] blood[1]	Fresh, platelet-rich plasma *or* platelet concentrate	1 litre Estimated deficit
Fibrinogen concentration	0·10 g./100 ml. plasma	Purified fibrinogen	Estimated deficit (see text)
Thromboplastin generation screening test			
(*a*) Without kaolin	4·0 × prolongation[2] at 3 mm. min. 1·5 × prolongation at 6 min.	Fresh citrated plasma	1 litre in ¾ hr.
(*b*) With kaolin	2·0 × prolongation at 2 min. 1·2 × prolongation at 4 min.		

[1]Thrombocytopenia need not be corrected in obstetrical emergencies if delivery is by the natural route; or even, perhaps, for surgery where abnormal bleeding or bruising, or purpura, have not already occurred, and provided other haemostatic defects can be made good, although it would be wise to have platelets 'on short call'.
[2]This indicates the multiplication factors by which the subsample clotting times from the patient's plasma reaction mixture are prolonged over those from the control plasma reaction mixture at the sampling times indicated. These critical values correspond to about 30% of the normal A.H.G. activity, with factor V normal (assume this if Quick's prothrombin time is normal).

expected from a consideration of the dose given and of the patient's blood volume, and if the subsequent reading is still lower, it may be concluded that the fibrinogen-consuming process is still active *in vivo*.

RELATIVE VALUE OF THE TESTS The essence of this communication is that in the acute bleeding states, multiple haemostatic defects may be found, and that for the analysis of these conditions the pathologist therefore requires a group of tests which, between them, will detect the various abnormalities which have been encountered.

Nevertheless it may not always be possible to make all the tests proposed; and for these occasions it is suggested that the three most important would be the platelet count, the fibrinogen estimation or the thrombin clotting time, and the thromboplastin generation screening test. If all these are normal, an important defibrination or fibrinolytic process can be excluded; if, however, these first are abnormal, the other techniques should be made to identify the type of abnormality which is present.

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Studies on the blood lipids and lipoproteins in thalassaemia and sickle cell anaemia

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SYNOPSIS In Cooley's anaemia the serum lipid fraction, determined by chemical analysis, was found to be decreased. The lipoprotein distribution by paper electrophoresis showed a characteristic pattern with abnormally low α_1 fraction and high concentrations of the β fractions. There is no relationship between age and serum lipoprotein distribution. A correlation was found between the electrophoretic pattern, liver function tests, and the E/C index. Similar changes but of lesser degree were found in sickle cell anaemia. Liver damage is thought to be the probable cause of the reduced α_1 fraction.

Previous studies from this clinic and from other centres (Allamanis, 1955; Panizon, 1957) have demonstrated disturbances in the electrophoretic pattern of the blood proteins in thalassaemia major and sickle cell anaemia consisting of a slight to moderate fall of the albumin fraction, a raised γ globulin fraction, and abnormal liver function tests. The relative value of these findings led us to study the lipid metabolism (Ackermann, Toro, and Kountz, 1954; Rafstedt, 1955; Salt and Wolff, 1957) of chronic haemolytic anaemias.

The sera of 32 patients with severe Cooley's anaemia, of nine patients with sickle cell anaemia, and of six patients with miscellaneous types of anaemia were studied. Twenty-seven normal cases of similar ages served as controls. Serum, 1·3 ml., was used for the determinations: Total lipids were determined by the Kunkel method (Kunkel, Ahrens, and Eisenmenger, 1948) using 0·2 ml. serum. Total cholesterol and its esters were calculated by Bloor's method and lipid phosphorus by the method of Fiske and Sabbarow. The lipid phosphorus was multiplied by the factor 25 to give the total phosphatides. (These two determinations together required 1 ml. serum.) A slight modification of the micromethod described by Swahn (1953) was employed to determine the relative concentrations of lipid in electrophoretically separated lipoprotein fractions, using 0·1 ml. serum. All sera were obtained from the patient in the fasting state before any blood transfusion had been given and examined in the next 48 hours.

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RESULTS

GROUP I: CONTROLS The findings are reported in Table I and Fig. 1. The results are within normal limits.

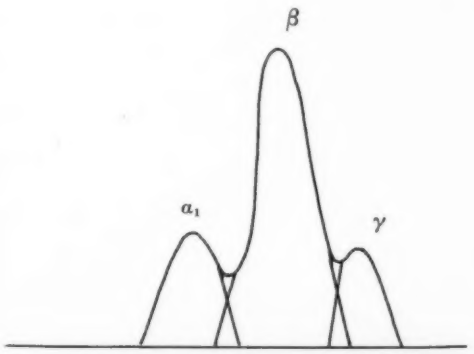

FIG. 1. *Electrophoretic pattern and levels of lipid fractions in normal controls.*

TABLE I

MEAN VALUE OF LIPID FRACTIONS AND LIPIDOGRAM IN 27 NORMAL CASES

Lipidogram			Total Lipid	Cholesterol	Esterified Cholesterol	$\frac{E}{C}$	Lipid Phosphorus or Phosphatides	$\frac{\beta}{\alpha_1}$
α_1	β	γ						
Ten Adults								
30·7	59·55	8·97	554	180	118	0·63	6·22 or 155 mg. %	1·93
Six children aged 6 to 11 years old								
30·94	59·27	9·69	461	163	114	0·73	5·43 or 136 mg. %	1·91
Seven children aged 2 to 6 years								
30·11	59·09	10·62	436	160	104	0·72	7·13 or 178 mg. %	1·96
Four babies aged 0 to 2 years								
31·38	57·27	11·19	476	122	96	0·78	6 or 150 mg. %	1·82

TABLE II

MEAN VALUE OF LIPID FRACTIONS AND LIPIDOGRAM IN 32 CASES OF COOLEY'S ANAEMIA

Lipidogram			Total Lipid	Cholesterol	Esterified Cholesterol	$\frac{E}{C}$	Lipid Phosphorus or Phosphatides	$\frac{\beta}{\alpha_1}$	Remarks
α_1	β	γ							
Normal lipidogram									
35·07	52·98	11·9	326	125	89	0·71	5·4 or 135 mg. %	1·42	Child 0 to 2 years old
39·99	53·54	9·95	358	110	53	0·48	6·58 or 165 mg. %	1·34	Child > 6 years old
Moderate decrease of α_1 fraction									
27·89	59·46	12·59	395	117	73	0·63	4·84 or 121 mg. %	2·11	0 to 2 years old, 2 cases
27·12	62·48	10·37	385	136	69	0·51	3·94 or 98·5 mg. %	2·30	> 6 years old, 4 cases
Definite decrease of α_1 fraction									
23·29	66·03	10·55	361	152	72	0·47	6·17 or 154 mg. %	2·83	0 to 2 years old, 2 cases
23·49	63·37	13·12	449	187	99	0·54	5·32 or 133 mg. %	2·71	2 to 6 years old, 6 cases
21·26	70·13	8·59	240	100	38	0·38	3·95 or 99 mg. %	3·29	> 6 years old, 1 case
Very low α_1 fraction									
18	70·34	11·54	374	120	61	0·51	5·01 or 125 mg. %	3·90	0 to 2 years old, 7 cases
17·27	70·71	12	335	118	61	0·52	4·15 or 104 mg. %	4·09	2 to 6 years old, 5 cases
14·78	73·79	11·41	329	140	76	0·53	3·66 or 91 mg. %	4·99	> 6 years old, 3 cases

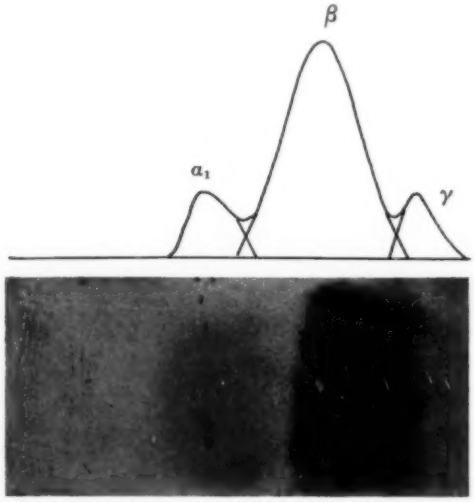

FIG. 2. *Electrophoretic pattern and levels of lipoprotein in patients with Cooley's anaemia.*

GROUP II: COOLEY'S ANAEMIA (Table II, Fig. 2) Th serum of all lipid fractions determined by chemic analysis was found to be decreased. The mean value of the total serum lipids was 357 mg. %. The reduction of esterified cholesterol was greater than of cholesterol so that the mean valueof the index E/C = $0{\cdot}52\left(\frac{\text{esterified cholesterol}}{\text{cholesterol}}\right)$. The mean value of the phosphatides was 122 mg. %. The paper electrophoretic pattern of lipoproteins showed that α_1 lipoproteins were decreased whereas the β fraction was increased (Table II and Fig. 2). Only two cases have a normal electrophoretic pattern of lipoproteins, In six cases the percentage value of the α_1 fraction was moderately decreased, in nine cases definitely decreased, and in 15 cases greatly decreased. In the majority of cases (19 out of 28), the reduction of the α_1 fraction of lipoproteins was associated with a decreased E/C index and positive flocculation tests. On the remaining nine cases, the E/C index and flocculation tests were normal in six cases, in two cases the E/C index was increased while the flocculation tests

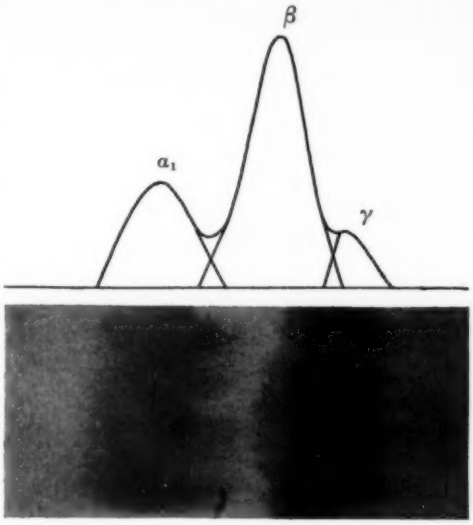

FIG. 3. *Electrophoretic pattern and levels of lipoprotein in patients with sickle cell anaemia.*

were normal, and in one with a positive flocculation test the E/C index was within normal limits.

GROUP III: SICKLE CELL ANAEMIA Although all fractions of blood lipids were seen to be reduced, the reduction was of smaller degree than in Cooley's anaemia.

The lipidogram showed minimal changes. The α_1 fraction was slightly decreased. In five patients the moderate reduction of the α_1 fraction was associated with a decreased E/C index and abnormal liver function tests. In three other patients with normal flocculation tests the E/C index was normal (two cases) or decreased (one case), but in another case positive flocculation tests were associated with a normal E/C index. These findings are illustrated in Table III and Fig. 3.

GROUP IV: MISCELLANEOUS In three cases of iron-deficiency anaemia, two of congenital spherocytosis, and one of haemolytic anaemia (Marchiagava Micheli) the α_1 fraction of lipoproteins was found to be within normal limits (Table IV).

DISCUSSION

In Cooley's anaemia all lipid fractions determined by chemical analysis were found to be decreased. The lipoprotein distribution by paper electrophoresis showed a characteristic pattern with diminished α_1 values and increased β values. Similar changes but of lesser degree were observed in sickle cell anaemia.

Our findings suggest that there is no relationship between age and serum lipoprotein distribution.

From the available literature the view could be upheld that the characteristic electrophoretic pattern with an abnormal α_1 fraction and the reduction of all lipid fractions is due to the liver damage which is found in advanced cases. This hypothesis is supported when the above findings are correlated with the abnormal liver tests and the reduced E/C index.

TABLE III

MEAN VALUE OF LIPID FRACTIONS AND LIPIDOGRAM IN SICKLE CELL ANAEMIA

Lipidogram			*Total Lipid*	*Cholesterol*	*Esterified Cholesterol*	$\frac{E}{C}$	*Lipid Phosphorus or Phosphatides*	$\frac{\beta}{\alpha_1}$	*Remarks*
α_1	β	γ							
25·25	61·03	12·91	375	135	62	0·40	5·66 or 141 mg. %	2·41	2 to 6 years old, 2 cases
27·77	60·58	11·60	386	144	71	0·52	5·15 or 129 mg. %	2·19	> 6 years old, 7 cases

TABLE IV

MEAN VALUE OF LIPID FRACTIONS AND LIPIDOGRAM IN MISCELLANEOUS CASES

Lipidogram			*Total Lipid*	*Cholesterol*	*Esterified Cholesterol*	$\frac{E}{C}$	*Lipid Phosphorus or Phosphatides*	$\frac{\beta}{\alpha_1}$
α_1	β	γ						
Three cases of iron-deficiency anaemia								
29·74	60·40	10·18	409	144	71	0·49	6·22 or 167 mg. %	2·30
Two cases of spherocytic anaemia								
36·04	54·99	8·96	367	111	53	0·55	3·31 or 94 mg. %	1·52
One case of haemolytic anaemia (Marchiafava Micheli)								
40·50	49·30	10·18	433	184	96	0·52	5·24 or 131 mg. %	1·21

However, some of the results are not readily explained.

There is no relationship between age and serum lipoprotein distribution and reduced lipid fractions, though severity of the disease increases with advancing age. Moreover, in some cases there is no correlation between the reduced a_1 fraction, flocculation tests, and E/C index.

In view of the above findings further studies are needed to determine if the liver damage is due to the anaemia alone or has its onset in early life and its severity is simply exaggerated by the progress of the chronic haemolytic process.

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Hereditary elliptocytosis in two Maltese families

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SYNOPSIS The findings in two elliptocytic families are recorded and compared. It is the first report of the anomaly in the Maltese population.

A variable degree of clinical and haematological expression among the members of the two families has been observed, ranging from healthy individuals with normal cell morphology to others with only elliptocytic erythrocytes and mild anaemia.

Two subjects have been studied during anaemic crises, and are considered to represent the homozygous state.

The phenomenon of elliptocytosis was first observed by Dresbach in 1904, and since then several reports of its occurrence in various races have been published. The anomaly is transmitted as a Mendelian dominant (Cheney, 1932; Strauss and Daland, 1937) linked with the Rh genes but is unrelated to sex (Goodall, Hendry, Lawler, and Stephen, 1953, 1954; Lawler and Sandler, 1954; Marshall, Bird, Bailey, and Beckner, 1954; Morton, 1956; Clarke, Donohoe, Finn, McConnell, Sheppard, and Nicol, 1960). The factors which induce the erythrocytes to assume an oval form at the late reticulocyte stage in this condition are unknown.

Wyandt, Bancroft, and Winship (1941) reviewed 246 cases reported in the literature and recorded their observations in 86 cases. They supported the general view that hereditary elliptocytosis represents a harmless abnormality unassociated with any significant disturbances. This view has been modified since Penfold and Lipscomb (1943) estimated from the number of cases in the literature that at least 12% of individuals bearing the anomaly manifest signs of increased destruction of red cells. Red cell survival studies published since show that the elliptocytes of patients with haemolytic anaemia have a shortened life span both when transfused into normal recipients (Lipton, 1955) and also when reinjected into the patient (Josephs and Avery, 1955; Blackburn, Jordan, Lytle, Swan, and Tudhope, 1958; Dacie, 1960).

The clinical and laboratory findings in two unrelated Maltese families are reported in this paper. Two members of one of the families are believed to represent the homozygous state.

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FAMILY S

The pedigree of family S is shown in Fig. 1, and the laboratory findings of the members examined in Table I.

CASE IV.10 A baby boy was well till the age of 4 months when he was admitted to hospital for gastroenteritis and was then given a blood transfusion. At the age of 17 months he was again referred to the hospital for pallor and splenomegaly. The blood findings at the time (20 June 1956) are included in Table I. There were no Leishman-Donovan bodies in the splenic pulp and the long bones were normal radiologically. The anaemia was not improved with iron and vitamin supplements. On 17 April 1957 he was admitted to hospital for further investigation because of slight icterus and persisting anaemia and splenomegaly.

Laboratory findings The haemogram on 22 April 1957 and other data determined subsequently are shown in Table I.

The blood smear (Fig. 2) showed that 70% of the red cells were elliptocytes and 24% microspherocytes; some of the elliptocytes were large and others very small, while rod-shaped, tailed, and bizarre forms were common, and schistocytes were also present. Target cells were not found at any time. Diffuse basophilia and normoblasts were absent. Sickle cells were absent in sodium metabisulphite preparations; no abnormal haemoglobin was detected by paper electrophoresis. The direct Coombs test was negative, and no abnormal antibodies were detected in the serum. The bone marrow showed pronounced erythroid hyperplasia (M.E.R.: 0·94/1.; L.E.R.: 0·73/1.). The saline osmotic fragility on 22 April was increased: initial lysis was at 0·72% NaCl (normal control at 0·44%) and was

TABLE I

LABORATORY FINDINGS FOR EACH MEMBER OF FAMILY S

No.	Sex	Date	Age	Haemoglobin (g. per 100 ml.)	R.B.C. (m. per c.mm.)	P.C.V. (%)	M.C.V. (cu.)	M.C.H.C. (%)	Elliptocytes (%)	Spherocytes (%)	Reticulocytes (%)	Osmotic Fragility (% NaCl) Initial	Osmotic Fragility (% NaCl) Complete	Serum Bilirubin (mg. per 100 ml.)	Foetal Haemoglobin (%)	Blood Groups ABO	MNS	Rhesus	P	Lea	Leb	Kell	Lutheran
III. 5[1]	M	9/ 5/57	47	12·2	4·0	38	95	32	98	Few	4·8	0·48	0·30	1·2	2·4	0	MsNs	R_1R_2	—[4]	—	—[4]	—	—
III.10[1 3]	F	9/ 5/57	45	12·3	3·7	37	100	33	30		2·0	0·46	0·30	0·6	1·4	0	MSMs	rr	+	—	+[4]	—	—
IV. 1[1]	M	9/ 5/57	23	13·1	4·3	40	93	33	70		2·8	0·44	0·30	0·5	2·4	0	MsNs	R_1r	—[4]	—	+	—	—
IV. 2[1]	M	27/ 5/57	22	13·0	5·0	38	76	34	Few		1·2	0·42	0·30	0·98	0·4	0	MsNs	R_1r	+[4]	—	—	—	—
IV. 3[1]	F	9/ 5/57	20	11·3	3·5	35	100	32	100	Occasional	3·4	0·42	0·30	1·2	2·3	0	MSMs	R_1r	—[4]	—	+	—	—
IV. 4[1]	F	27/ 5/57	18	11·3	3·8	35	92	32	100		3·4	0·44	0·30	2·2	1·1	0	MsMs	R_1r	—[4]		—	—	—
IV. 5[1]	F	9/ 5/57	17	11·7	3·7	35	95	33	100	Occasional	0·8	0·42	0·30	1·0	5·2	0	MsNs	R_1r	—[4]	—	+	—	—
IV. 6[1]	F	9/ 5/57	12	12·9	4·7	39	83	33			2·0	0·46	0·30	0·7	0·9	0	MsNs	R_1r	—[4]	-	—	—	—
IV. 7[1]	F	9/ 5/57	11	12·4	4·4	37	84	34	97	3	3·8	0·46	0·30	0·5	3·9	0	MsMs	R_1r	—[4]	+	—	—	—
IV. 8[1]	M	9/ 5/57	10	12·1	4·7	35	75	35	61		0·4	0·46	0·30	1·0	1·5	0	MSNs	R_1r	—[4]	—	+	—	—
IV. 9[1]	M	9/ 5/57	7	9·8	3·8	29	76	34	92	Occasional	2·6	0·48	0·30	1·0	3·0	0	MSMs	R_1r	—[4]	—	+	—	—
		27/ 5/59		5·0	2·9						1·1												
		1/ 6/59									11·6												
		2/ 6/59		7·1							8·4												
		5/ 6/59		9·0							12·0												
		6/ 6/59		8·3	3·9	26	67	32	87	8	4·5												
		9/ 6/59		9·2							5·7												
		24/ 6/59		11·1							3·3												
IV.10[1 5]	M	20/ 6/56	2	9·0	5·0				82	11						0	MSMs	R_1r	+	+	—	—	—
		22/ 4/57		9·7	4·7	32	68	30	70	24	4·2	0·72	0·36	1·2	1·7								
(Imferon)		30/ 8/57		8·1					45	40	8·9			1·5									
(Imferon)		10/ 9/57		9·0					33	31	7·9												
(Imferon)		19/ 9/57		8·7	4·4																		
		3/10/57		9·0	4·4	31	70	29	49	25	3·8	0·60	0·28	1·8	1·6								
		18/ 1/58		8·6					53	20	10·4												
		5/ 4/58		7·3	4·1	27	66	27	59	36	13·3			0·8	0·7								
		17/ 5/58		9·3	4·4				45	35	4·0												
		4/ 4/59		9·9	4·6	30	65	33	64	34	7·0	0·60	0·30	1·5	2·2								
		20/ 5/59		4·6	2·0	13	65	35	52	35	0			0·9									
IV.11[1]	F	17/ 8/59	19 hr.	13·9	5·6	47	84	30	32	20	7·4				47·0	0		R_1r					
		28/ 8/59	11 days	12·6	5·1				52	9	1·1												

Direct Coombs test: all negative. [3]Sickling test: negative. [2]No Rh antibodies in the serum. [3]No abnormal antibodies detected in the serum. [4]Some doubt in the

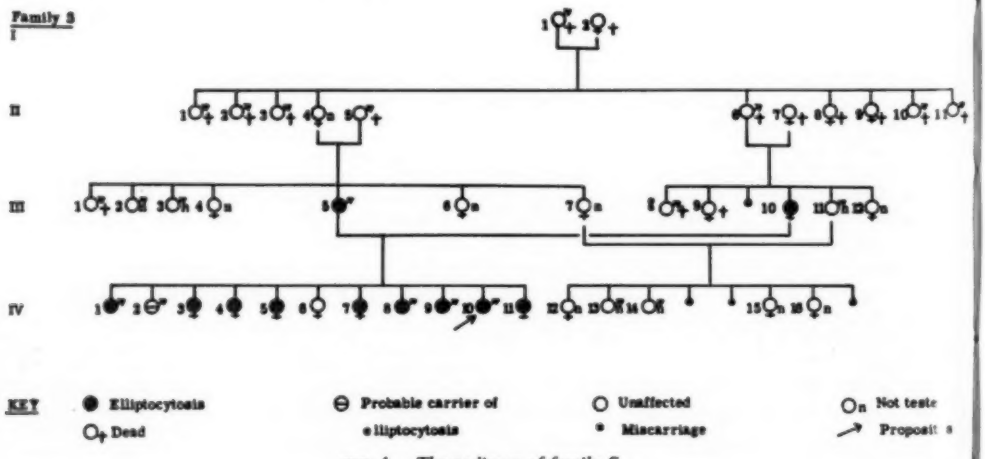


FIG. 1. *The pedigree of family S.*

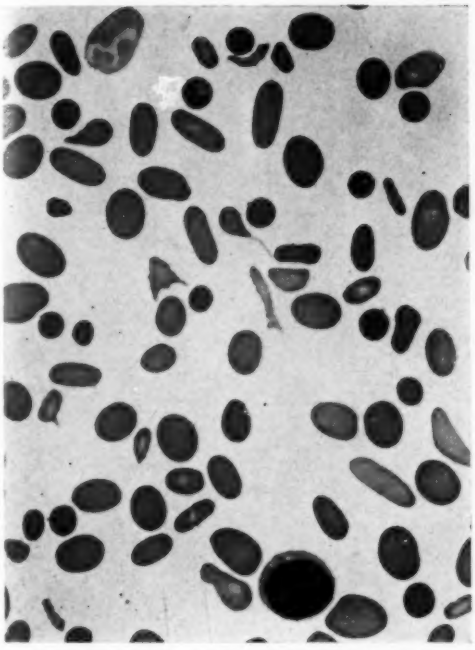


FIG. 2. *Photomicrograph of a peripheral blood film made from Case IV.10 of family S* (× 900).

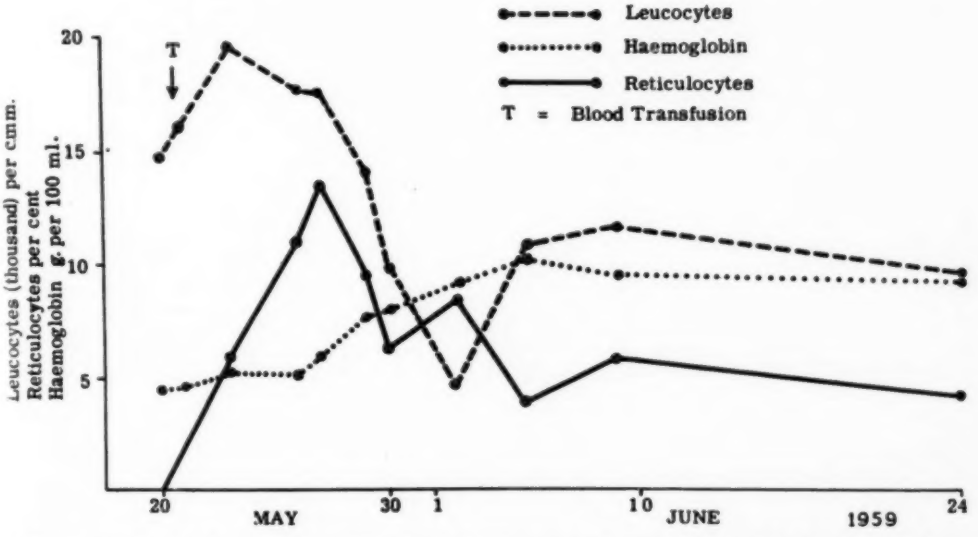
complete at 0·36% NaCl (normal control at 0·30%). The plasma bilirubin level was 1·2 mg. per 100 ml. and excess urobilin was present in the urine. Turbidity tests of liver function and the serum electrophoretic pattern were normal.

Course Oral iron was given but soon had to be suspended because the child started to vomit after each dose. The child's condition improved slightly, and he was followed up as an out-patient. In April 1958 he was treated for otitis media, and there was no remarkable change in his condition then.

In an attempt to improve the anaemia, two courses of intramuscular iron (Imferon) were administered at an interval of 16 months without benefit. He was re-investigated in April 1959 and no significant changes found.

In May 1959, he was taken ill with fever (T. 101°F.), increased pallor and polylymphadenitis, and was re-admitted to hospital nine days after the onset. The physical findings on admission were: temperature 100°F.; pulse rate 144 per minute; respiration 40 per minute; scanty rales at both lung bases; spleen palpable 4 cm. below costal arch; enlarged soft mobile lymph nodes in neck, axillae, and groins.

The relevant haematological findings at this time are listed in Table I. Blood culture, serum agglutination against *Salm. typhi* and *Br. melitensis*, Paul Bunnell test, and Sabin's dye and toxoplasma fixation tests were all negative. He was transfused with 150 ml. group O Rh-positive blood, and given penicillin 500,000 units daily. The temperature resolved by lysis, and the child was afebrile after 10 days' treatment. The changes in the haemoglobin, reticulocyte, and leucocyte counts recorded during this period are shown in Fig. 3.



G. 3. *Curves showing changes in leucocyte and reticulocyte counts and haemoglobin level in Case IV.10 during an plastic' crisis.*

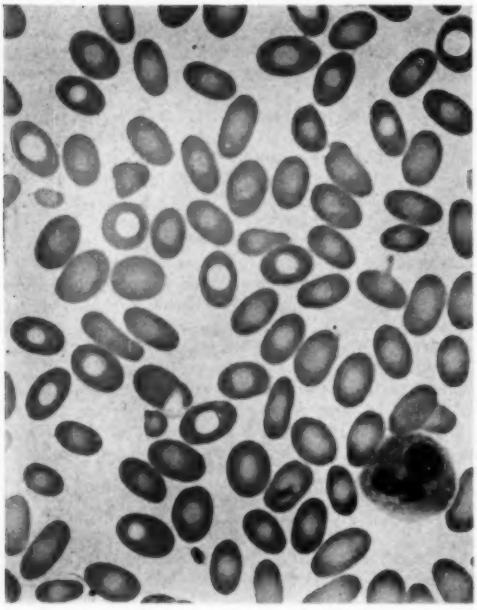


FIG. 4. *Photomicrograph of a peripheral blood film made from the father of Case IV.10 of family S* (× 900).

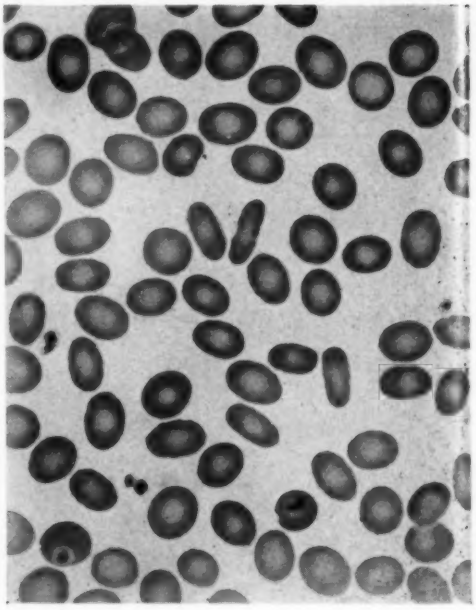


FIG. 5. *Photomicrograph of a peripheral blood film made from the mother of Case IV.10 of family S* (× 900).

Family study Both parents are of Maltese stock and are first cousins. They and all their progeny have been investigated. There is no history of abortions or stillbirths.

Both parents have elliptical erythrocytes in blood films, and are mildly anaemic. Almost all the erythrocytes of the father (Fig. 4) are elliptocytic or ovalocytic. The number of elliptocytes in the mother (Fig. 5) is much smaller. Only two of the siblings (IV.2, IV.6) do not show evidence of elliptocytosis; the remaining siblings have an elliptocyte count ranging between 30 and 100%. Evidence of increased red cell destruction was found in some of the members. The direct Coombs test was negative in both parents and siblings, while no Rhesus antibodies were detected in the mother's serum. No abnormal haemoglobin was detected by paper electrophoresis but six of the members have a significant increase in Hb F levels.

The family history of both parents was unrevealing.

FAMILY K

The pedigree of family K is shown in Fig. 6. In Table II are recorded the haematological findings of the members investigated.

CASE V.13 A boy, the third in the family, was healthy until the age of 5 years when he suffered from bronchitis which responded to terramycin, and a few weeks later he developed measles. This in turn was followed by a period of low-grade pyrexia, and pallor was noticed for the first time. He was referred to the out-patient department for investigation and on examination no physical signs, apart from pallor, were detected. Routine blood examination showed elliptocytes.

Laboratory findings The haemogram is recorded in Table II. The blood smear (Fig. 7) showed definite elliptocytes and ovalocytes, and only rare microspherocytes. There was marked anisocytosis, but bizarrely shaped erythrocytes were scanty. There were no target cells. Basophilic stippling was present but no normoblasts.

The direct Coombs test was negative. No abnormal Hb was detected by electrophoresis, and the Hb F level was well within normal. The bone marrow was not examined.

Course The child was followed up for a period during which there was no significant alteration in the haematological findings, except for the presence of spherocytes and poikilocytes in blood films, which were not a feature in previous films. The liver was palpable 3 cm. below the costal arch at this time. He has been in apparent good health since.

Family study The mother of the propositus is of Maltese stock, while one of the father's ancestors is of English extraction. Of the eight pregnancies, two (the fourth and the seventh) terminated in an abortion, and the sixth was a stillbirth.

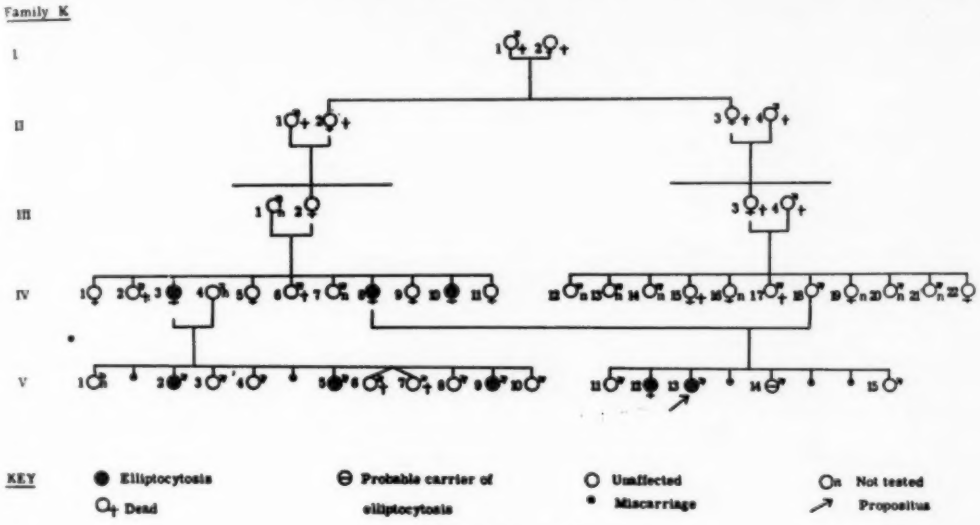


FIG. 6. *The pedigree of family K.*

TABLE II

LABORATORY FINDINGS FOR EACH MEMBER OF FAMILY K

Sex	Date	Age	Haemoglobin (g. per 100 ml.)	R.B.C. (m. per c.mm.)	P.C.V. (%)	M.C.V. (μ³)	M.C.H.C. (%)	Elliptocytes (%)	Spherocytes (%)	Reticulocytes (%)	Osmotic Fragility (% NaCl) Initial	Osmotic Fragility (% NaCl) Complete	Serum Bilirubin (mg. per 100 ml.)	Foetal Haemoglobin (%)	Blood Groups ABO	MNS	Rhesus	P	L^{a}	L^{b}	Kell	Lutheran	Duffy
F	10/12/57	65	18·0					Rare							O	MS	rr	+[a]	–	+	–	–	–
F	10/12/57	41	15·0					Rare							O	MN	rr	+	–	–	–	–	–
F	10/12/57	38	14·7					24							B	MNS	rr	–	–		–	–	–
F	10/12/57	34	16·2					Occasional							B	MN	rr	+	–		–	–	–
F	4/12/57	26	14·4	4·6	42	91	34	57		2·0	0·46	0·30	0·5	1·0	O	MSNS	rr	+	–	–	–	–	–
F	10/12/57	24	18·0					Rare							B	MN	rr	–	–		–	–	–
F	10/12/57	21	14·7					57							B	MN	rr	+	–		–	–	–
F	10/12/57	18	16·2					Rare							O	MN	rr	+	–	+	–	–	–
M	14/12/57	27	15·6	5·0	46	92	34	Occasional		1·0	0·46	0·30	0·3	0·8	A_1	MsNs	R_1r	–	–	–	+	+	–
F	10/12/57	18	15·0					Occasional							A_1	MsNs	R_1r	+	–	+[a]	+	–	–
M	22/ 1/58	13	9·5	3·6	31	86	33	78	Rare	2·6	0·46	0·30	0·5	1·0	B	M	R_1r	–	–		+	–	–
	26/ 2/59		10·9	3·8						4·6													
M	24/ 2/58	11	15·7					Rare		2·3	0·46	0·30	0·3		A_2B	Mi	R_1r	–	–		–	–	–
M	24/ 2/58	10	14·3					Rare		0·8	0·44	0·30	0·3		B	MN	R_2r	–	–		–	–	–
M	24/ 2/58	9	12·0					85		2·0	0·46	0·30	0·3		O	M	R_2r	–	+	–	–	–	–
M	24/ 2/58	5	14·3							1·2	0·46	0·30	0·5		O	M	R_2r	–	+	–	–	–	–
M	24/ 2/58	3	12·5					85		1·1	0·46	0·30	0·3		B	MN	R_1r^s	–	+		–	–	–
M	24/ 2/58	2	12·9							1·6	0·44	0·30	0·3		A_2B	MN	R_1r	–	+		–	–	–
M	4/12/57	7	14·1	4·6	41	89	34	Rare		1·3	0·46	0·30	0·2	1·1	A_1	NSNs	R_1r	–	–	–	+	–	–
F	4/12/57	6	14·1	5·0	42	84	34	23		2·5	0·44	0·30	0·3	1·8	A_1	MSMs	R_1r	+	–	–	–	+	–
M	4/12/57	5	11·7	4·7	39	83	30	100		4·3	0·44	0·30	0·7	1·4	A_1	NSNs	R_1r	–	–	–	+	–	–
	3/ 1/58		11·6							1·6													
	2/ 8/58		12·1	4·4				84	4														
M	4/12/58	3	14·4	5·8	43	74	34	10		1·8	0·44	0·30	0·5	1·1	A_1	NSNs	R_1r	+	+	–	–	–	–
M	5/ 8/58	2 mth.	11·3	3·7	35	95	32	6		2·1	0·46	0·30	0·5	35·0	A_1		R_1r						
	5/10/58	4 mth.	11·1	4·9				3		1·1													

test negative. [a]Some doubt in the grouping.

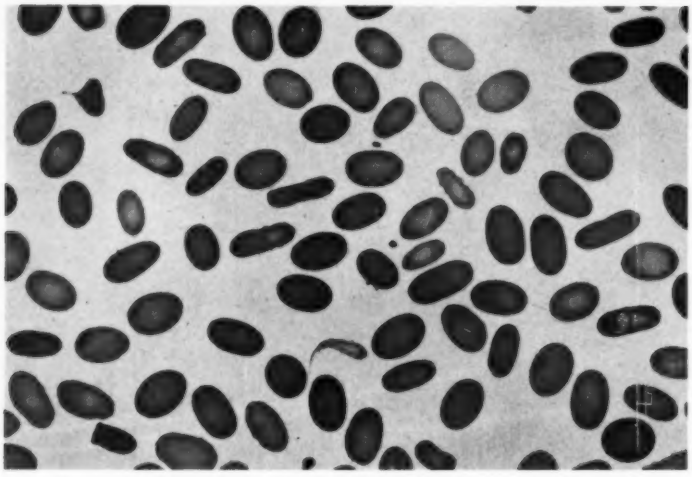


FIG. 7. *Photomicrograph of a peripheral blood film made from Case V.13 of family K* (× 900).

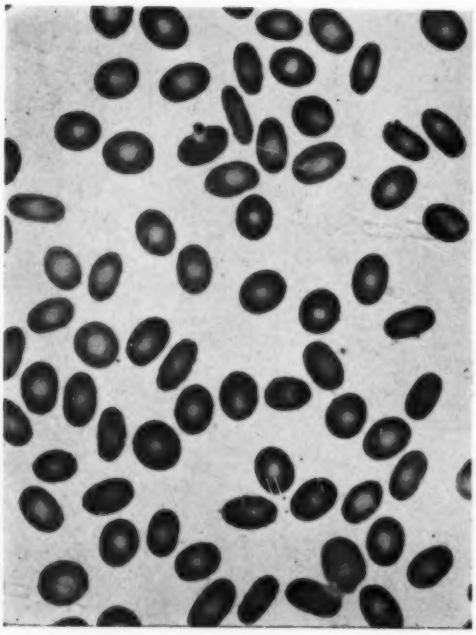


FIG. 8. *Photomicrograph of a peripheral blood film made from the mother of Case V.13 of family K* (× 900).

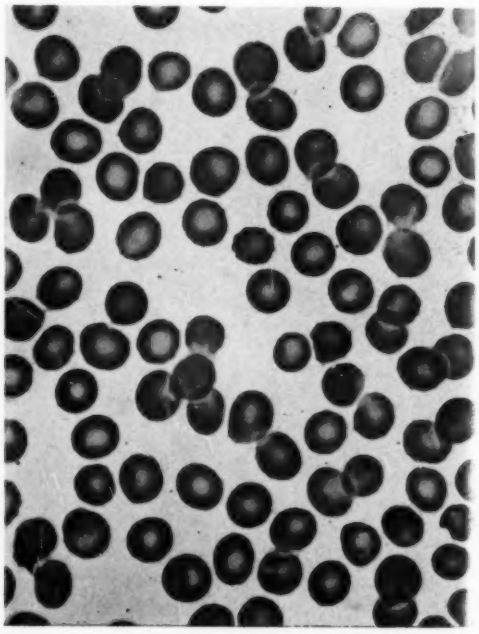


FIG. 9. *Photomicrograph of a peripheral blood film mad from the father of Case V.13 of family K* (× 900).

The peripheral blood film (Fig. 8) of the mother (IV.8) and that of another of her children (V.12) show elliptocytosis, but that of the father (IV.18) shows only round cells (Fig. 9). Only the propositus is anaemic; both affected siblings show a mild reticulocytosis.

In all, 22 members of family K have been investigated. The other branch of the family (V.1 to 10) are the children of unrelated parents. There are two abortions in this family, and a twin pregnancy terminated prematurely, both infants dying in the third month, following a respiratory infection. The blood of three of the seven siblings examined shows definite elliptocytosis, and that of a fourth shows a preponderance of round cells with an appreciable number of oval rather than elliptical forms. Anaemia was present in two of the three affected siblings. The reticulocyte count was raised in one of the affected siblings who is also suffering from rheumatic endocarditis, and in his brother who has oval but no elliptical cells.

Three of the members in sibship IV.1 to 11 showed the anomaly but none were anaemic. The maternal grandmother and a paternal aunt of the propositus were normal.

DISCUSSION

CRITERIA FOR DIAGNOSIS Several criteria have been proposed for the diagnosis of elliptocytosis (Günther, 1928; Hedenstedt, 1947; Florman and Wintrobe, 1938; Lambrecht, 1938) but the work of Wyandt *et al.* (1941) indicates that 0·2% of normal subjects have between 10 and 15% oval red cells in their peripheral blood. Among the 35 members in the two families under study, 17 have an elliptocyte count ranging between 30 and 100%; in two the count is between 20 and 25%, and in the remaining 16, elliptocytes are either absent or number less than 1%. We believe that an elliptocyte count above 20% in any subject whose near relatives show unquestionable evidence of elliptocytosis constitutes the full carrier state. On this basis 11 members in family S and eight members in family K are full carriers.

GENETIC CONSIDERATIONS Both parents (III.5 and III.10) in family S carry the anomaly and are first cousins. We were able to find one report (Lipton, 1955) in the literature of such an occurrence, and possibly a second was one of the cases reported by Lendvai (1949). Wyandt *et al.* (1941) encountered the anomaly in both parents in one instance, but there was no consanguinity. The only living grandparent (II.4) has refused to be examined. Of the 11 children in this sibship, only two have round cells, and one of them (IV.2) does show a few elliptocytes.

The parents in family K too are related but it is only the mother who shows the anomaly. The father and one of his sisters have only round cells. Two of the five siblings show the anomaly; the elliptocyte count of the propositus (V.13) is 100%, that of the other sibling (V.12) is 23%. Yet another brother has 10% elliptocytes and may well represent the 'occult' trait. In sibship V.1 to 10, three members have 78 to 85% elliptocytes. Three of the seven antecedents examined in sibship IV.1 to 11 have an elliptocyte count ranging between 24 and 57%.

We could not produce any evidence for linkage of the gene for elliptocytosis with any of the Rh genes, both parents in both families being homozygous for the latter genes. Our observations agree with previous reports on the absence of sex linkage, and we were also unable to detect associated skeletal abnormalities (Günther, 1928; Gallais, Collomb, and Miletto, 1956; Barnett and Brown, 1957), chronic leg ulceration (Evans, 1943), or a reduction in the elliptocyte count in succeeding generations (McCarty, 1934).

HAEMATOLOGICAL STUDIES In family S, IV.9 and IV.10 are suffering from overt haemolytic anaemia, while other members (III.5, III.10, IV.3, IV.4, and IV.5) have a mild degree of anaemia and macrocytosis (Dacie, Mollison, Richardson, Selwyn, and Shapiro, 1953; McBryde, Hewlett, and Weisman, 1956). The slightly raised reticulocyte counts and serum bilirubin levels are evidence of a compensated haemolytic process (Holst-Larsen, 1947).

Only one member (V.2) out of the 22 examined in family K is anaemic, but the anaemia may be due to the rheumatic endocarditis rather than to the elliptocytosis. Slightly raised reticulocyte counts were, however, found in the majority of the elliptocyte members but the serum bilirubin levels in the same subjects were normal (Wyandt *et al.*, 1941; Kirkegaard and Larsen, 1942; Holst-Larsen, 1947.) The blood of unaffected members, with one exception, have a somewhat raised Hb value, together with a mild macrocytosis in blood films. Similar observations have been previously recorded by Stephens and Tatelbaum (1935), Wyandt *et al.* (1941), and by Blackburn *et al.* (1958).

The lower Hb values in our series were invariably associated with elliptocytosis and normal haematocrit indices, except in the two subjects with manifest haemolytic anaemia.

Spherocytic microcytes and micro-elliptocytes (Fig. 2) were found in significant numbers in only one instance. This finding has been reported both before (Lipton, 1955) and after splenectomy (Wilson and Long, 1953). Splitting of erythrocytes of an infant has been observed under the microscope (Lipton, 1955), and microspherocytes in elliptocytic haemolytic anaemia may originate by this process.

We have, in agreement with previous observers, noted that the change in contour occurs at the late reticulocyte stage. It has been our experience, however, that the elliptocyte count is appreciably lower

in brilliant cresyl blue preparations than it is in Romanowsky-stained films.

Strauss and Daland (1937) noted that the saline osmotic fragility was normal in the absence of anaemia. Our findings lend support to this view though a marked increase in fragility was observed in one of the proposita (IV.10, family S).

Sicklaemia and hereditary elliptocytosis may coexist (Evans, 1943; Fadem, 1949; Charles and Suitters, 1959; Clarke *et al.*, 1960). The test for sickling was negative in 23 of the members in our series. We have also failed to detect any abnormal Hb by paper electrophoresis.

Normal Hb F levels are reported to be a feature of elliptocytosis by Motulsky, Singer, Crosby, and Smith (1954), Wilson and Long (1953), Lipton (1955), Plissier, Mechali, Delons, and Fulerand (1957) but Singer, Chernoff, and Singer (1951), and White and Beaven (1954) recognize the occasional detection of traces of Hb F. We have detected a slight increase above the accepted normal value in six members in family S. Haemoglobin F values did not correlate with the elliptocyte count, although the lowest values recorded in family S are those of the two non-elliptocytic members.

ELLIPTOCYTOSIS IN INFANCY It was Hunter (1932) who first reported the presence of elliptocytes in infancy. It is now established that the anomaly may be manifest at birth and the elliptocyte count reaches a peak at about the third month of post-natal life. (Hunter, 1932; Wyandt *et al.*, 1941; Helz and Menten, 1944). In Lipton's case (1955) hereditary elliptocytosis was complicated by haemolytic anaemia as early as the first month. Similar observations were made by Josephs and Avery (1955).

One member in our series (V.15, family K) was studied at the age of 2 months. A mild anaemia, but only a small number of oval erythrocytes were present. At 4 months the number of oval cells was even less. We have also studied another member (IV.11, family S) 19 hours after birth. In contrast to the former infant, her Hb level was low at this time and lower still subsequently, while the elliptocyte count rose from 32% to 52% within 11 days. This change was accompanied by a sharp fall in the reticulocyte count. We have been prevented from following the infant further, but there was already enough evidence to show that she, like most of her family, is a full carrier.

ELLIPTOCYTOSIS AND ANAEMIC CRISES The association of elliptocytosis with haemolytic anaemia is now well established in the literature. In general, it appears that an infection often provokes a crisis. Another complication which is perhaps less well recognized is the development of an 'aplastic' crisis. We have been able to study concomittantly two members in family S (IV.9 and IV.10) during such an episode. Both presented clinically and haematologically the same features. Reticulocytes were absent in the peripheral blood, while immature granulocytes, including myeloblasts, were present. As the reticulocyte count steadily rose, the immature granulocytes disappeared. In one instance (IV.10), the child was transfused but his brother (IV.9) recovered spontaneously.

CLINICAL VARIATION IN ELLIPTOCYTIC FAMILIES The findings in the two families presented suggest the following grouping: (*a*) Normal, only round erythrocytes present; (*b*) occult trait, erythrocytes mainly round and elliptocytes less than 20%; (*c*) elliptocytosis, elliptocytes over 20%, no haemolysis; (*d*) elliptocytic haemolytic anaemia (compensated), elliptocytes over 20%, anaemia not present, minor degrees of haemolysis; (*e*) elliptocytic haemolytic anaemia (uncompensated), elliptocytes over 20%, continuous anaemia, anaemic crises. The distribution in the two families is shown in Table III.

Admittedly the classification of any one member is not as obvious and easy as may be inferred. The distinction between the normal and the occult trait is somewhat arbitrary, and can only be deduced by comparing the findings with the overall picture within the family. The compensated forms of elliptocytic haemolytic anaemia present some features suggesting the uncompensated forms, and minor degrees of anaemia may be due to other secondary causes. We have thus adopted a somewhat conservative approach in our grouping. It can be readily seen from our data that in only a few members is the distinction sharply demarcated.

It is, however, significant that nine of the 11

TABLE III

DISTRIBUTION OF CLINICAL VARIATION IN THE TWO FAMILIES

	Normal	*Occult Trait*	*Elliptocytosis*	*Elliptocytic Haemolytic Anaemia (compensated)*	*Elliptocytic Haemolytic Anaemia (uncompensated)*	*Total*
Family S	1	1	5	4	2	13
Family K	13	1	5	2	1	22
Total	14	2	10	6	3	35

children of affected consanguineous parents definitely show the anomaly, and that two of them have presented with haemolytic or 'aplastic' crises during the period of our study. The latter two siblings (IV.9 and IV.10) have been considered to represent the homozygous state. This seems to be an exceptionally rare occurrence. In the case reported by Wyandt *et al.* (1941) the parents were not related, and in Lipton's case (1955) the relationship of the parents was more distant than that reported here.

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The significance of Howell-Jolly bodies and giant metamyelocytes in marrow smears

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SYNOPSIS Howell-Jolly bodies in erythroid precursors can be found in the marrow in a variety of diseases. More than 1% in the later erythroblasts is rarely found except in vitamin B_{12} or folic acid deficiency states.

Giant metamyelocytes are probably always the result of vitamin B_{12} or folic acid deficiency. A distinction is drawn between the deficiency of, and therapeutic need for, vitamin B_{12} and folic acid.

The detection of vitamin B_{12} or folic acid deficiency is often difficult, sometimes impossible, by examining marrow alone, particularly if the alteration in the nuclear pattern of the erythroblasts is the only pathology on which the diagnosis is to be made. Cox, Matthews, Meynell, Cooke, and Gaddie (1960) refer to the many reports of patients with vitamin B_{12} deficiency and normoblastic erythropoiesis. Where methods of vitamin B_{12} and folic acid estimation are available the interpretation of ambiguous erythropoiesis may assume less importance, but these procedures are not yet suitable for routine hospital work. The report of Hutchison and Ferguson-Smith (1959) on the correlation between Howell-Jolly bodies in red cell precursors and vitamin B_{12} or folic acid deficiency is thus of importance, but we considered it advisable to confirm their findings, especially in cases where the type of erythropoiesis was difficult to interpret. At the same time we decided to follow Lehmann's (1955) suggestion and review the significance of giant metamyelocytes in the marrow.

Other features generally considered to be associated with a megaloblastic change are premature haemoglobinization and a maturation arrest of the erythroblasts. The former is a most subjective assessment and may be valueless in the presence of concomitant iron deficiency, and was therefore not considered. The degree of maturation arrest had to be taken into account when determining the significance of Howell-Jolly bodies and the value of this feature is also discussed.

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MATERIALS AND METHODS

Marrow smears from the following conditions (numbers of cases in brackets) were examined.

GROUP I CONDITIONS RESPONDING TO VITAMIN B_{12} OR FOLIC ACID Pernicious anaemia (45), post-gastrectomy anaemia (5), malabsorption syndrome (5), nutritional B_{12} deficiency (1), leukaemia with megaloblastic anaemia (2), and anticonvulsant macrocytic anaemia (6). Marrow examinations were made on these patients before the start of treatment, and, in addition, 12 patients with megaloblastic anaemia under treatment were investigated.

GROUP II CONDITIONS WITHOUT APPARENT B_{12}, FOLIC ACID, OR IRON DEFICIENCY Leukaemia (10), other myeloproliferative disorders (12), carcinoma (8), thrombocytopenic purpura (8), chronic infection (7), refractory normoblastic anaemia (3), cirrhosis (3), haemolytic anaemia (2), various miscellaneous disorders (7), and normal (8). There were 22 cases of iron-deficiency anaemia not following upon a gastrectomy.

The features assessed in each smear were (1) the percentage of erythroid precursors earlier than the intermediate stage of development. Three hundred erythroblasts were counted and the percentage calculated to the nearest whole number; (2) the number of Howell-Jolly bodies in 500 erythroblasts, and the presence of two or more Howell-Jolly bodies in any of these erythroid precursors. Howell-Jolly bodies were not seen in cells earlier than the intermediate stage of development. Therefore, because of the varying degree of maturation arrest in the two groups, the counts were corrected, using the percentage of erythroid precursors to give the percentage of Howell-Jolly bodies in the intermediate or late erythroblasts. Cells in mitosis were excluded from the counts in Nos. 1 and 2 above. (3) The type of erythropoiesis, as judged on 500 cells, was classified as normoblastic, megaloblastic if any cell showed the

characteristic nuclear change, and transitional megaloblastic if any cell showed a transformation towards a megaloblast but none had reached this degree of abnormality. (4) Cells of the metamyelocyte or stab stage larger than 18μ were counted as giant metamyelocytes. They were searched for under a high-power objective in squash preparations of marrow particles giving highly cellular fields. Their frequency in the smears was recorded as follows:—

0=none in whole smear
+=1 to 5 in whole smear
++=several in whole smear, but less than one per four fields
+++=1 or more per four fields

RESULTS

The findings in group I (excluding cases under treatment) and group II are presented in Table I.

TABLE I

INCIDENCE OF HOWELL-JOLLY BODIES, GIANT METAMYELOCYTES, AND ERYTHROID PRECURSORS IN CASES WITH (GROUP I) AND WITHOUT (GROUP II) APPARENT VITAMIN B_{12} OR FOLIC ACID DEFICIENCY

	Group I (64)	*Group II*	
		Without Iron-deficiency (68)	*Iron-deficiency Anaemia* (22)
Howell-Jolly bodies (%)			
0	0	39	5
1	3	25	12
1-2	6	2	2
2-3	7	1	2
3+	48	1	1
Smears showing erythroblasts containing multiple Howell-Jolly bodies	60	5	2
Giant metamyelocytes			
0	2	64	12
+	15	3	7
++	26	0	2
+++	21	0	1
Erythroid precursors (%)			
Normal	17	62	18
Increased	47	6	4

Figures under groups refer to number of cases.

HOWELL-JOLLY BODIES In group I, Howell-Jolly bodies were found in every smear. In 96% there were 1% or more, and cells containing two or more bodies were found in 94%. In contrast, although 50% of the cases in group II contained Howell-Jolly bodies, only 10% had over 1% and 8% had multiple bodies in any cell. The highest counts of Howell-Jolly bodies were in group I, 32·4%, and in group II, 5·4%. The degree of anaemia did not account for the difference in findings between the two groups. In group II there was no correlation between the presence or the number of Howell-Jolly bodies and the severity of the anaemia whereas in group I there was a relationship, although it was not close. Separating the cases according to size of the Howell-Jolly bodies gave no useful information.

There was no significant difference in the percentage of Howell-Jolly bodies between those conditions in group I that responded to B_{12} and those that responded to folic acid (six anticonvulsant macrocytic anaemias, three malabsorption syndrome, two leukaemias).

Among the eight normal marrows, Howell-Jolly bodies were found in three, though each had less than 1%. In none were multiple bodies present.

GIANT METAMYELOCYTES In group I giant metamyelocytes were found in 97% of cases, and in large numbers (++ or +++) in 73%. They were not seen in two cases. In group II they were seen in 14% of the cases, and in large numbers in 3%. The three cases of group II without iron deficiency in which giant metamyelocytes were seen were two with chronic infections and one with acute myeloid leukaemia. No giant metamyelocytes were seen in the normal marrow smears.

The more severely anaemic patients in group I usually had the more numerous giant metamyelocytes, but, as with Howell-Jolly bodies, the correlation between the degree of anaemia and numbers found was not close. There was no such correlation in the group II cases in which they were present. There was no difference in the frequency of giant metamyelocytes between the cases which responded to B_{12} and those which responded to folic acid.

PERCENTAGE OF ERYTHROID PRECURSORS From previous experience a figure of up to 9% for this finding was considered to be normal. The eight normal cases in group II fell below this level. In group I, 73% and in group II, 11% showed an increase in the proportion of erythroid precursors: the highest percentage in group I was 50 and in group II 37. In group I there was a fairly close correlation between the percentage of erythroid precursors and the severity of the anaemia.

From these results the features suggestive but not diagnostic of B_{12} or folic acid deficiency appeared to be 1% or more cells containing Howell-Jolly bodies or multiple bodies in any erythroblast; several giant metamyelocytes in smear (++ or more); 10% or more early erythroid precursors.

Of the 19 cases in group II with one or more of these features, 18 were not considered to be deficient in vitamin B_{12} or folic acid for the following reasons: Ten cases (nine iron-deficiency anaemias and one pyridoxine-responsive anaemia) responded promptly and completely to appropriate therapy without

administration of vitamin B_{12} or folic acid. Three cases (two refractory normoblastic anaemias and one pre-leukaemic leukaemia) did not respond to vitamin B_{12} or folic acid. Five cases (one each of congestive cardiac failure, cirrhosis, idiopathic acquired haemolytic anaemia, Hodgkin's disease, and polycythaemia rubra vera) gave no clinical or haematological indications of vitamin B_{12} or folic acid deficiency during the subsequent course of their disease. One patient with acute myeloid leukaemia (a smear showed 1·4% and multiple Howell-Jolly bodies) died soon after marrow puncture and therefore without adequate follow-up. In this case a deficiency of vitamin B_{12} or folic acid cannot be excluded with certainty.

APPLICATION OF RESULTS TO DIFFICULT MARROWS

Supportive evidence of vitamin B_{12} or folic acid deficiency in the marrow smear is only needed in cases where the megaloblastic transformation is absent or incomplete. The value of this evidence can only be judged if it is of assistance in such instances, and the cases without frankly megaloblastic marrows in group I were therefore considered separately (Table II). In the 23 cases 1% or more Howell-Jolly bodies were present in 21, multiple Howell-Jolly bodies in 16, giant metamyelocytes (++ or more) in 14, and the percentage of erythroid precursors increased in eight. One or more of these changes were, however, present in every case.

The problem of the investigation of an anaemia after vitamin B_{12} or folic acid has been given to the patient is not infrequent. Return of megaloblastic to normoblastic erythropoiesis is rapid and corroborative evidence of previous vitamin B_{12} or folic acid deficiency persisting after the erythroblastic reversion would be valuable. To determine how long the abnormalities considered here remain after treatment a series of marrows taken at varying times after the start of vitamin B_{12} or folic acid therapy was examined (Table III). The persistence of supportive evidence might depend on the degree of initial change and the pre-treatment findings in the eight cases in which they were available are also shown. There is an insufficient number of cases from which to draw definite conclusions. Cases 10 and 11 may have received suboptimal therapy and the results may thus be misleading. In the others, after erythropoiesis was no longer frankly megaloblastic, Howell-Jolly bodies and the percentage of erythroid precursors were still abnormal in two, and giant metamyelocytes in three (with an occasional one in three more).

IRON-DEFIENCY ANAEMIA

Originally all the cases in group II were considered together but it was noticed that many of them showing the features suggestive of vitamin B_{12} or folic acid deficiency were cases of iron-deficiency anaemia. Smears from patients with this disease were therefore

TABLE II

TWENTY-THREE CASES WITHOUT FRANKLY MEGALOBLASTIC MARROWS RESPONDING TO VITAMIN B_{12} OR FOLIC ACID THERAPY

Case No.	*Hb (g.%)*	*Howell-Jolly Bodies (%)*	*Multiple Howell-Jolly Bodies*	*Giant Metamye-locytes*	*% Erythroid Precursors*	*Erythro-poiesis*	*Diagnosis*
1	14·6	6·6	Yes	+++	13	T.M.	Pernicious anaemia
2	14·0	2·2	Yes	+	9	N.	S.A.C.D.
3	12·6	1·8	No	++	11	T.M.	Pernicious anaemia
4	12·5	1·6	No	+	3	N.	S.A.C.D.
5	12·0	8·0	Yes	++	4	T.M.	Pernicious anaemia
6	12·0	2·4	Yes	++	10	T.M.	Pernicious anaemia + S.A.C.D.
7	11·3	4·8	Yes	+	9	T.M.	Pernicious anaemia + S.A.C.D.
8	11·2	4·2	Yes	0	4	T.M.	Pernicious anaemia
9	11·2	4·0	Yes	+++	8	T.M.	Idiopathic steatorrhoea
10	11·0	2·0	No	+	6	T.M.	Nutritional vitamin B_{12} deficiency
11	11·0	4·0	Yes	++	8	T.M.	Chronic lymphatic leukaemia
12	10·6	1·0	No	++	15	T.M.	Pernicious anaemia
13	10·0	4·6	No	+	13	T.M.	Pernicious anaemia
14	9·6	1·4	No	+++	9	T.M.	Idiopathic steatorrhoea + iron-deficienc
15	9·5	2·6	No	++	16	T.M.	Post-gastrectomy + iron-deficiency
16	9·4	5·8	Yes	+	5	T.M.	Pernicious anaemia
17	9·4	2·0	Yes	+	7	T.M.	Pernicious anaemia + S.A.C.D.
18	8·4	0·4	Yes	+++	8	T.M.	Post-gastrectomy + non-reactive tuber culosis
19	8·0	3·6	Yes	+++	18	T.M.	Pernicious anaemia + chronic pylonephriti
20	7·7	1·8	Yes	++	8	T.M.	Post-gastrectomy + iron-deficiency
21	7·0	4·4	Yes	++	9	T.M.	Anticonvulsant macrocytic anaemia
22	5·6	0·8	Yes	++	6	T.M.	Pernicious anaemia (? spontaneous remis sion)
23	5·6	5·6	Yes	0	10	T.M.	Acute lymphatic leukaemia

recorded separately in Table I, and those with giant metamyelocytes, excess Howell-Jolly bodies, or a raised percentage of erythroid precursors in more detail in Table IV. These 12 showed no constant features either in their marrow findings or clinical data, and in the latter respect did not differ from the 10 whose marrows had no evidence suggestive of vitamin B_{12} or folic acid deficiency.

SPLENECTOMY

Because of the known relationship between Howell-Jolly bodies in the circulating erythrocytes and splenectomy, the opportunity was taken to examine certain post-splenectomy marrows. Three patients had undergone splenectomy during upper abdominal operations two to three weeks previously. In each smear Howell-Jolly bodies were present, but less than 1% in number, and in none were they multiple. Two patients who now presented with pernicious anaemia had undergone splenectomy six and eight years ago during repair of hiatus hernia and for traumatic rupture. They showed 9·0% and 5·4% Howell-Jolly bodies, and both had cells with multiple bodies. These percentages were of the same order as the figures recorded in patients with pernicious

TABLE III

MARROW FINDINGS IN 12 PATIENTS RESPONDING TO VITAMIN B_{12} OR FOLIC ACID

	Before Treatment						*After Treatment*						*Diagnosis*	*Treatment*
Case No.	*Hb (g.%)*	*Erythropoiesis*	*Howell-Jolly Bodies (%)*	*Multiple Howell-Jolly Bodies*	*Giant Metamyelocytes*	*% Erythroid Precursors*	*Interval between Start of Treatment and Marrow Aspiration*	*Erythropoiesis*	*Howell-Jolly Bodies (%)*	*Multiple Howell-Jolly Bodies*	*Giant Metamyelocytes*	*% Erythroid Precursors*		
24	5·6	M	32·4	Yes	+++	25	15½ hrs.	M	32·8	Yes	+++	21	Pernicious anaemia	Cytamen intramuscularly
25	4·6	—	—	—	—	—	22 hrs.	M	4·2	Yes	+++	43	Pernicious anaemia	Cytamen intramuscularly
26	4·6	M	7·2	Yes	+++	26	3 days	N	0·8	Yes	+++	6	Pernicious anaemia	Cytamen intramuscularly
27	7·2	—	—	—	—	—	5 days	T.M.	6·2	Yes	++	9	Pernicious anaemia	Cytamen intramuscularly
28	4·9	—	—	—	—	—	8 days	T.M.	0·8	No	++	16	Pernicious anaemia	Cytamen intramuscularly
29	5·6	—	—	—	—	—	10 days	N	0·2	No	+	3	Pernicious anaemia	Cytamen intramuscularly
6	12·0	T.M.	2·4	Yes	++	10	14 days	N	—	No	+	4	Pernicious anaemia	Cytamen intramuscularly
30	7·0	M	5·0	Yes	+++	31	14 days	N	0·2	No	0	6	Pernicious anaemia	Cytamen intramuscularly
31	7·0	M	0·8	Yes	++	15	15 days	N	—	No	+	10	Pernicious anaemia	Cytamen intramuscularly
32	7·0	M	16·8	Yes	++	11	18 days	N	—	No	0	8	Pernicious anaemia	Folic acid
10	11·0	T.M.	2·0	No	+	6	21 days	N	1·4	Yes	0	6	Nutritional vitamin B_{12} deficiency	Oral B_{12}
11	11·0	T.M.	4·0	Yes	++	8	28 days	N	1·0	No	0	5	Chromic lymphatic leukaemia	Folic acid

TABLE IV

TWELVE CASES OF IRON-DEFICIENCY ANAEMIA RESPONDING TO IRON THERAPY WITH MARROW FINDINGS SUGGESTIVE OF VITAMIN B_{12} OR FOLIC ACID DEFICIENCY

Case No.	*Sex*	*Age*	*Hb (g.%)*	*M.C.H.C. (%)*	*Marrow Findings*				*Free Acid in Test Meal*	*Recent Iron Therapy before Marrow Examination*	*Duration of Symptoms*	*Aetiology of Anaemia*
					Howell-Jolly Bodies	*Multiple Howell-Jolly Bodies*	*Giant Metamyelocytes*	*% Erythroid Precursors*				
69	M	64	10·8	—	1·0	No	+	4	—	Yes	Weeks	Peptic ulcer, chronic bronchitis
70	M	66	9·2	26	2·0	Yes	0	3	—	No	Weeks	Idiopathic
72	F	80	8·6	27	2·0	Yes	+	10	Present	Yes	Years	Steatorrhoea, duodenal diverticulum
73	F	83	8·2	29	0·4	No	+++	9	—	Yes	Months	Hiatus hernia
74	M	63	8·0	24	5·4	Yes	+	11	—	Yes	Weeks	Peptic ulcer, chronic bronchitis
75	F	72	7·4	27	0·6	No	+	6	—	Yes	Years	Idiopathic
77	M	40	6·8	24	0·2	No	+	6	Absent	Yes	Years	Haemorrhoids
78	F	33	6·4	—	0·2	No	++	9	Present	No	Weeks	Idiopathic
79	F	45	5·8	22	0·6	No	+	7	Absent	No	Years	Duodenal ulcer
81	F	52	5·8	—	0·2	No	++	17	Present	No	Years	Idiopathic
84	F	46	5·5	—	1·2	No	0	2	—	Yes	Months	Menorrhagia
89	M	59	3·6	—	0·2	No	+	10	Absent	No	Months	Gastric ulcer

anaemia with similar degrees of anaemia who had not undergone splenectomy. A sixth patient with a refractory normoblastic anaemia showed 1·0% Howell-Jolly bodies before and 0·6% 11 months after splenectomy.

DISCUSSION

HOWELL-JOLLY BODIES IN ERYTHROID PRECURSORS Hutchison and Ferguson-Smith (1959) in a study of the significance of the presence of nuclear fragments in the erythroid precursors considered it probable that disordered nucleic acid synthesis resulting from vitamin B_{12} or folic acid deficiency was the fundamental factor in their production. They did, however, record four cases in which infrequent solitary inclusions were found but in which there was no evidence of vitamin deficiency, and they thought that the bodies were possibly artefacts. They also found similar inclusions in one case of iron-deficiency anaemia.

It might be argued that the inclusions found in our cases without vitamin deficiency were not in fact Howell-Jolly bodies, but this we think unlikely. Inclusions were only counted if they were of the same degree of staining as the nucleus of the parent cell. Often in grossly megaloblastic marrows the bodies show the same reticulation as the cell nucleus. Pappenheimer bodies for which they might be mistaken are usually of a lighter hue than the nucleus and occur often in greater numbers in one cell than Howell-Jolly bodies (other than in grossly megaloblastic cells). Howell-Jolly bodies are not refractile, a feature which distinguishes them from some artefacts. In megaloblastic cells in which there were many bodies of all sizes, the small ones were identical to the inclusions found in scanty numbers in non-vitamin deficiency cases.

Since occasional Howell-Jolly bodies occur in normal marrows and in conditions in which deficiency of B_{12} or folic acid can be excluded, a quantitative assessment of their presence would appear to be essential if the finding is to be of value when most needed. This count takes only a few minutes to perform and at the same time permits an accurate assessment of erythropoiesis. Where erythropoiesis is obviously megaloblastic a Howell-Jolly count is of course superfluous.

Our findings show that a count of less than 1% of Howell-Jolly bodies has no significance as regards the aetiology of the anaemia, but that more than this number or the presence of multiple bodies in one cell is highly suggestive but not pathognomonic of vitamin B_{12} or folic acid deficiency. The value of the finding of excess Howell-Jolly bodies in difficult marrow smears is shown in the selected cases of Table II, where an increased number was found in 7/1, and multiple bodies in 16, of the 23 smears. If assay methods of vitamin B_{12} and folic acid levels are not available, an excess number of Howell-Jolly bodies is sufficient evidence to justify a therapeutic trial of vitamin B_{12} or folic acid. Unfortunately, in cases where therapy has been started a search for Howell-Jolly bodies is of little value since they rapidly disappear with the conversion of erythropoiesis.

GIANT METAMYLELOCYTES The literature on the specificity of giant metamyelocytes is conflicting. Whitby and Britton (1957) quote Henning (1935) to the effect that they are typical of pernicious anaemia but note that Jones (1943) met them in a variety of diseases. Jones, however, had found them as well as in pernicious anaemia only in diseases associated with megaloblastic transformation of the marrow, namely, achrestic anaemia, tropical macrocytic anaemia, sprue, and pernicious anaemia of pregnancy. Scott (1939) commented on their presence in one or two of 23 cases of iron-deficiency anaemia. Foy, Kondi, and Hargreaves (1950) considered them to be as pathognomonic as megaloblasts in those anaemias which respond to liver, folic acid, Marmite, or a high-protein diet. Later, Foy and Kondi (1952) reported that they were present in 20% of the marrows of their patients with hypochromic anaemia. Davidson (1954) in a survey of 500 marrows found them associated with iron-deficiency anaemia, leukaemia, and other diseases (not stated) as well as pernicious anaemia, and Lehmann (1955) quotes Davidson as finding giant metamyelocytes in 63 of 164 iron-deficiency anaemias. Tasker (1959) found some abnormal myeloid cells in many normoblastic iron-deficiency anaemia marrows which responded to iron therapy. MacIver and Back (1960), on the other hand, stated that they had never seen abnormal myeloid cells in iron-deficiency anaemia.

Because of these differing reports which suggest that giant metamyelocytes might not be confined to vitamin B_{12} or folic acid deficiency, we made a quantitative assessment of their presence in the marrow smears. This showed a definite relationship between the presence of many giant metamyelocytes and the deficiency of one of the vitamins. The three iron-deficiency anaemias containing many giant metamyelocytes would seem to refute this relationship, but this is not necessarily so since the work of Cox *et al.* (1960) and Cox, Meynell, Gaddie, and Cooke (1959) provides evidence of tissue deficiency if vitamin B_{12} in apparently straightforward iron-deficiency anaemia. Their cases responded satisfactorily to iron therapy alone, as ours did.

It appears therefore that the findings of frequent giant metamyelocytes is, in most cases, an indicat on

for vitamin B_{12} or folic acid therapy. Foy *et al.* (1950) and MacIver and Back (1960) have reported the response to folic acid of normoblastic anaemias with giant metamyelocytes though the frequency of such cells was not recorded. We have met several normoblastic anaemias in pregnancy with numerous giant metamyelocytes which responded to folic acid.

The value of finding numerous giant metamyelocytes in difficult smears appears to be less than that of Howell-Jolly bodies since only 14 of the 23 cases listed in Table II had such numbers but this is still a sufficiently common finding to make their assessment of value. But their absence does not exclude the possibility that the anaemia will respond to vitamin B_{12} or folic acid (Cases 8 and 23, Table II).

In cases already on vitamin therapy the number of giant metamyelocytes like Howell-Jolly bodies rapidly declines, although marrows eight days after the start of treatment may still contain many and an occasional one may be found two weeks later (Table III). These times are somewhat longer than the survival of giant metamyelocytes recorded by Leitner (1949).

Davidson (1952) commented that in iron-deficiency anaemia the giant metamyelocytes had nuclei almost, if not quite, as complicated as those associated with pernicious anaemia. Our impression was that regardless of the aetiology of the anaemia the more bizarre forms were found in those marrows with numerous giant metamyelocytes but in a few instances even the occasional one had an abnormal nucleus and is probably evidence of tissue depletion of vitamin B_{12} or folic acid. It is significant that of the 25 cases recorded here with occasional giant metamyelocytes, 15 responded to B_{12} or folic acid (two of these were normoblastic anaemias) and the other 10 were of iron-deficiency anaemia (see below) or had active myeloid marrows. It may be that in cases with increased myeloid activity this cell series, rather than the erythroid mass, would show the first overt signs of deficiency of B_{12} or folic acid. Spray and Witts (1953) have shown that patients with leukaemia appear to require abnormally large amounts of folic acid, though this is not a constant finding. Swendseid, Swanson, Meyers, and Bethell (1952) suggested that the folic acid depletion in leukaemia results from the production of large numbers of immature white cells of high folic acid content.

PERCENTAGE OF ERYTHROID PRECURSORS An arrest in maturation of the erythroid precursors was only prominent in frankly megaloblastic anaemias in group I, and as an aid to the diagnosis of difficult cases it is of little value. This is in accord with the experience of others (de Gruchy, 1958).

IRON-DEFICIENCY ANAEMIA The literature on giant metamyelocytes in iron-deficiency anaemia has been reviewed above. Our results support the findings of those authors who have recorded them to be a common occurrence in iron-deficiency anaemia. An increase in Howell-Jolly bodies is also not uncommon and is probably of similar significance, although it is not clear why the two abnormalities were not always present together. Of interest in this connexion is an analysis of the cases of vitamin B_{12} or folic acid deficiency in Table II, in which it is seen that the highest percentage was not necessarily associated with the most numerous giant metamyelocytes.

The finding of Cox *et al.* (1959, 1960) of low serum and marrow B_{12} levels in iron-deficiency anaemia has already been cited. There have also been reports of folic acid depletion in this disease (Spray and Witts, 1953; Chanarin, Mollin, and Anderson, 1958). We believe that the abnormal findings in our cases are the morphological results of one or more of these deficiencies. It is not our purpose to consider the possible ways in which iron deficiency may produce deficiency of B_{12} or folic acid but merely to record the frequency with which evidence of these deficiencies may be found.

The presence of such evidence in the marrow of a patient with iron-deficiency anaemia does not, however, mean that response to iron will be suboptimal or that the patient's anaemia will improve on vitamin B_{12} or folic acid. This has been our experience in anaemia of pregnancy. Cox *et al.* (1959) have shown that the low serum B_{12} level returns slowly to normal with iron therapy, further evidence of the relationship between the iron and vitamin deficiency. However, in certain circumstances, *e.g.*, anaemias associated with gastrectomy, the malabsorption syndrome, or pregnancy, giant metamyelocytes or excess Howell-Jolly bodies may be indications of a vitamin depletion concomitant with, but not resulting from, the iron deficiency, which will persist despite iron therapy. A case we have encountered since completing this series supports this contention. A patient with hypochromic normoblastic anaemia following partial gastrectomy with a serum B_{12} level of less than 50 $\mu\mu$g per ml. responded partly to parenteral iron but after 17 days the marrow had become frankly megaloblastic. The original marrow contained numerous giant metamyelocytes but no excess Howell-Jolly bodies. This change in erythropoiesis following iron therapy has been mentioned previously by Giles and Shuttleworth (1958) and Tasker (1959). Therefore, although giant metamyelocytes and, usually, an excess of Howell-Jolly bodies, are evidence of B_{12} or folic acid deficiency, only by knowing the indications for which marrow aspiration was performed can their clinical

significance be assessed. For example, if the patient has a mild normochromic anaemia with indeterminate erythropoiesis the finding of either abnormality is a valuable guide to the diagnosis of pernicious anaemia or related disease. On the other hand, such findings in an iron-deficiency anaemia, or in a marrow with myeloid hyperplasia, although basically due to vitamin B_{12} or folic acid depletion, indicate that haematological response to the appropriate therapy should be observed closely in case the vitamin deficiency should be unrelated to the primary disorder or irreversible without specific therapy.

SPLENECTOMY Our finding of Howell-Jolly bodies in the erythroid precursors in all our splenectomized cases is at variance with the report of Hutchison and Ferguson-Smith (1959). But we believe our results to be logical. If Howell-Jolly bodies were absent from the marrow in splenectomized patients one would have to postulate that the inclusions seen in the erythrocytes are the remains of the nucleus and that these cells are indeed nucleated red cells. But the regular eccentricity of Howell-Jolly bodies in the red blood cells and their much smaller size than the nuclei of the late normoblasts in the marrow, without intervening sizes being evident, does not support this idea.

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The rate of blood loss from skin punctures during the Ivy bleeding time test

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SYNOPSIS The rate of blood loss from skin punctures during the performance of the Ivy bleeding time test has been measured by a simple technique in normal individuals, in patients without defects of the haemostatic or coagulation system, and in patients with known haemorrhagic disorders.

A wide range was found in normal individuals, but repeated tests on a single individual showed a smaller variation. Nearly half of the tests on patients with von Willebrand's disease, thrombocytopenia, 'capillary type' of bleeding, or haemorrhagic renal failure gave abnormally high rates of blood loss. Haemophilic, Christmas disease, and Dinedevan-treated patients gave low volumes and rates of blood loss.

A group of patients has been encountered in whom the bleeding time was normal but the rate of blood loss was increased. The majority of these had haemorrhagic symptoms and other evidence of a defective haemostatic or coagulation system. It is suggested that a consideration of the rate of blood loss in those patients with a normal bleeding time gives additional help in interpreting the Ivy test. A high rate may indicate the need for further investigation of the haemostatic and coagulation system.

During the performance of the Ivy bleeding time test (Ivy, Shapiro, and Melnick, 1935) it can be seen that there is considerable variation in the amount and rate of blood loss from the skin punctures in different patients. This is so even when care is taken to avoid puncturing superficial veins. The possibility was recognized that a consideration of this blood loss might prove a useful adjunct to the Ivy bleeding time as a test of haemostasis.

TECHNICAL METHODS

HAEMOGLOBIN ESTIMATIONS These were performed using an Eel photoelectric colorimeter and oxyhaemoglobin as described by Dacie (1956) (100% is equivalent to 14·8 g. %).

IVY BLEEDING TIME This was performed as described by Biggs and Macfarlane (1957), using a spring lancet set to a depth of 3 mm. and taking care to avoid superficial veins. The skin punctures were blotted every quarter of a minute with Whatman No. 1 filter paper from a roll of width 1 cm. The normal range was taken to be a mean (from three punctures) of up to six minutes.

MEASUREMENT OF VOLUME OF BLOOD LOST FROM SKIN PUNCTURES The blood was eluted from the roll of filter paper used for blotting the skin punctures by placing it in a 100 ml. stoppered conical flask containing 50 ml. of ammoniated '0·04%' distilled water and agitating on a rotary mixer for 30 minutes. After centrifugation the haemoglobin concentration in the supernatant was estimated with an Eel photoelectric colorimeter. When the amount of blood loss was very great an appropriate dilution had to be made before estimation.

Since haemoglobin estimations are normally performed on a 1 in 200 dilution of blood a reading of '100%' in the eluate (referred to above) corresponds to a volume of blood equal to 1/200 × 50 ml. or 250 c.mm. Thus each '1%' on the haemoglobin scale corresponds to 2·5 c.mm. of blood lost from the skin punctures. If the patient was anaemic a proportionate correction was applied, depending upon the haemoglobin concentration of the capillary blood, *i.e.*, volume of blood loss in c.mm.

$$= \text{'\%' of haemoglobin in eluate} \times 2{\cdot}5 \times \frac{100}{\text{patient's Hb\%}}.$$

Results have been expressed as c.mm. of blood lost. The rate of blood loss per minute per puncture was calculated by dividing the total volume by the sum of the bleeding time, in minutes, from the three punctures.

TESTS OF THE COAGULATION SYSTEM These, including platelet counts, Quick's one-stage prothrombin time, Lee and White's clotting time, Merskey's prothrombin

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consumption index, and Hess's tourniquet test, were performed as described by Biggs and Macfarlane (1957). The antihaemophilic globulin assay was that of Biggs (1957).

SELECTION OF SUBJECTS

NORMAL VOLUNTEERS These were drawn from the technical and medical staff over a period of two years. Forty-seven tests were made on 16 males and 43 tests on 17 females. None was known to have haemorrhagic symptoms.

PATIENTS WITHOUT HAEMORRHAGIC SYMPTOMS OR DISORDERS OF COAGULATION SYSTEM These patients were suffering from hypertension, renal disease, or urinary tract abnormalities but enquiry and examination gave no evidence of a haemorrhagic tendency. Also the bleeding time, coagulation time, prothrombin time, prothrombin consumption index, and platelet counts were normal. Twenty-seven tests were made on 25 patients.

VON WILLEBRAND'S DISEASE Patients included in this group had a prolonged bleeding time, normal platelet count, and either a low antihaemophilic globulin (AHG) level or there was a prolonged bleeding time in a close relative (parent, sibling, or child). Sixteen tests were made on seven patients.

'CAPILLARY TYPE' OF HAEMORRHAGIC STATE Patients included in this group had a prolonged bleeding time and normal platelet count, coagulation time, prothrombin time, and prothrombin consumption index. All had haemorrhagic symptoms affecting the mucous membranes. A conclusive family history of a bleeding tendency was not demonstrable. Eleven tests were performed on 10 patients.

THROMBOCYTOPENIA Patients included in this group had a platelet count of less than 100,000 per c.mm. in the absence of any other known defect of the coagulation system. The causes were idiopathic thrombocytopenic purpura, leukaemia, aplastic anaemia, nitrogen mustard treatment, and liver failure. Fifteen tests were performed on 12 patients.

RENAL FAILURE WITH HAEMORRHAGIC SYMPTOMS Patients in this group had persistently raised levels of blood urea but normal platelet counts, coagulation times, and prothrombin times. The bleeding time was usually prolonged and prothrombin consumption impaired. All had haemorrhagic symptoms affecting the skin or mucous membranes. Thirty tests were performed upon 20 patients.

HAEMOPHILIA AND CHRISTMAS DISEASE These patients were diagnosed or investigated by Dr. Rosemary Biggs. Eight tests were performed on seven haemophiliacs and two tests on two patients with Christmas disease.

DINDEVAN-TREATED PATIENTS Three patients were tested in whom the 'prothrombin and proconvertin' percentages were 5, 13, and 13·5 (Owren and Aas, 1951). They did not have haemorrhagic symptoms and the bleeding times were normal.

THROMBOCYTHAEMIA Two patients with platelet counts of 680,000 and 1,035,000 per c.mm. were tested. The primary conditions were polycythaemia rubra vera and chronic myeloid leukaemia. The bleeding times were prolonged in both cases.

ACUTE DEFIBRINATION SYNDROME An obstetric patient with a severe haemorrhagic diathesis, investigated by Dr. A. A. Sharp of the Radcliffe Infirmary, was tested on three occasions. The platelet counts at these times were 94,000, 318,000, and 335,000 per c.mm. The bleeding times were prolonged on each occasion. The whole blood clotting time was greater than six hours initially.

RESULTS

Table I shows the range and mean values for bleeding time and rate of blood loss from 20 repeated tests on a single individual (M.J.A.).

TABLE I

RESULTS OF 20 REPEATED BLEEDING TIME TESTS ON A SINGLE NORMAL INDIVIDUAL

Measurement	*Mean*	*Range*
Bleeding time (min.)	3·6	1·5 to 7·2
Rate of blood loss (c.mm./min.)	6·8	1·4 to 13·0

Table II shows the rates of blood loss found in a group of normal volunteers, patients without haemorrhagic symptoms, and in groups of patients with different haemorrhagic states. In general higher rates were found in patients with von Willebrand's disease, 'capillary type' of bleeding, thrombocytopenia, or haemorrhagic renal failure than in normal volunteers, patients without haemorrhagic states, or in patients with plasma coagulation defects. The few tests on patients with thrombocythaemia or acute defibrination also gave high rates of blood loss.

Brief details are given in Table III of 15 patients with a probable haemorrhagic tendency, normal bleeding times and platelet counts, but a rate of blood loss from skin punctures which was higher than that usually encountered in normal individuals

DISCUSSION

These results show that abnormally high rates of blood loss from skin punctures are common in those types of haemorrhagic states attributed to a capillary or platelet abnormality. Defects of the plasma coagulation factors, such as occur in haemophilia Christmas disease, or after treatment with Dindevan

TABLE II

RATE OF BLOOD LOSS FROM SKIN PUNCTURES IN C.MM. PER MINUTE

Group of Subjects	*Range*	*Mean*	*No. of Tests Over 25 c.mm./min.*	*No. of Tests Under 52 c.mm./min.*
Normal volunteers	1·0-161·0[1]	12·6	12	78
Non-haemorrhagic patients	1·5- 24·0	11·6	0	27
Haemophilia, Christmas disease, Dindevan-treated patients	1·2- 11·0	6·1	0	12
			12	117
von Willebrand's disease	3·6- 92·0	34·1	5	5
Capillary type of bleeders	7·5- 53·4	24·1	4	7
Thrombocytopenia	3·5- 50·0	19·0	4	11
Haemorrhagic renal failure	7·0-108·0	29·8	16	14
Thrombocythaemia	36·3-100·0	68·2	2	0
Acute defibrination syndrome	11·3- 88·0	41·5	2	1
			33	38

[1]The next highest value in this group was 47·6 c.mm./min.

TABLE III

GROUP OF PATIENTS WITH PROBABLE HAEMORRHAGIC STATES BUT NORMAL BLEEDING TIME AND PLATELET COUNTS

	No. of Patients	*Rates of Blood Loss from Skin Punctures (c.mm./min.)*
Menorrhagia + bruising or epistaxis	4	30, 32, 42, 80
Repeated haemorrhages after dental extractions	2	32, 40
Chronic nephritis + epistaxis ± bruising	3	31, 40, 108
Purpura while recovering from subacute bacterial endocarditis	1	55
Recovering from attack of idiopathic thrombocytopenic purpura	1	48
Haematemesis and melaena, hepatosplenomegaly	1	43
Gastrointestinal bleeding, ? 'capillary bleeder'	1	35
Excessive bleeding from skin trauma	1	55
Mother of a patient thought to have von Willebrand's disease	1	66
	15	Mean 48·7

do not appear to result in an increased rate of blood loss from skin punctures.

In addition a number of patients with suspected haemorrhagic states have been found to have a normal bleeding time but an unusually high rate of blood loss, *i.e.*, 30 c.mm. or more per minute. These 15 cases are listed in Table III. Haemorrhagic symptoms, present in all but one, were confined to the skin and mucous membranes and were generally mild in character. This clinical pattern of the bleeding suggested an abnormality of capillary or platelet function. In some cases it was partially confirmed by a positive tourniquet test or by a prolonged bleeding time on a different occasion. In another instance the patient's daughter had a long bleeding time. The four cases with renal failure all had impaired prothrombin consumption due to a qualitative platelet defect (Willoughby, 1960).

In the presence of a normal bleeding time the finding of a high rate of blood loss suggests defective cutaneous haemostasis where it might not otherwise be suspected. In the presence of a prolonged bleeding time it provides confirmatory evidence. Because of the imperfections of available tests of haemostasis this information proves useful in practice. The two tests are complementary to each other and the measurement of rate of blood loss cannot replace the bleeding time since, as shown in Table II, the mean rate is often within the normal range in patients with a long bleeding time.

The only other report that has been found in which consideration is given to the amount of blood loss is that of Adelson (1956). He reported that Dextran infusion in large amounts caused a haemostatic defect which could be demonstrated by using a 'modification of the Ivy technique whereby the bleeding was measured by volume of blood as well as by duration'. No figures for the volumes of blood were given. It is of interest that Dextran appears to produce this haemostatic defect by becoming adsorbed on to the surface of platelets (Rothman, Adelson, Schwebel, and Langdell, 1957) and

inhibiting their participation in the early stages of blood coagulation (Jacobaeus, 1955).

We should like to thank Dr. Rosemary Biggs for her advice and encouragement. She and the physicians and surgeons of the Oxford United Hospitals have kindly given access to the case notes of patients under their care. We should also like to thank the many members of the staff who volunteered for these tests. Some of the work reported here was included in a thesis submitted (by M.L.N.W.) to the University of London for the degree of Doctor of Medicine (1960).

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Methicillin-resistant staphylococci

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SYNOPSIS Eighteen strains of *Staph. pyogenes* (nine penicillin-sensitive and nine penicillin-destroying) were passaged 40 to 50 times on Celbenin[1] ditch plates.

All strains developed an increase in resistance to Celbenin and eight strains (four penicillin-sensitive and four penicillin-destroying) were able to grow in 100 μg/ml. or more Celbenin. Resistance was of the drug-tolerant type and none of the cultures inactivated Celbenin. There was an associated increase in tolerance to benzyl penicillin.

The highly Celbenin-resistant cultures isolated from penicillin-destroying staphylococci were in sharp contrast to those from penicillin-sensitive strains, as well as to penicillin G-tolerant staphylococci isolated *in vitro*, because they retained the cultural characteristics, coagulase and haemolytic activity, and mouse virulence of the parent strains, and the degree of resistance remained stable after repeated passage in the absence of Celbenin.

Three naturally occurring Celbenin-resistant strains of *Staph. pyogenes* isolated from infective processes were also studied. All three strains grew luxuriantly in concentrations of Celbenin up to 12·5 μg/ml. but very poorly in higher concentrations.

The possible significance of these findings is discussed.

The chemical isolation of the nucleus of penicillin, 6-amino penicillanic acid, followed by the preparation of a new penicillin, Celbenin (sodium 6-(2-6-dimethoxybenzamido) penicillinate), which is almost completely resistant to staphylococcal penicillinase (Rolinson, Stevens, Batchelor, Wood, and Chain, 1961), is probably the most interesting and important advance in the chemotherapy of staphylococcal infection since the discovery of penicillin itself. As is well known, staphylococci resistant to benzyl penicillin can be broadly divided into two types. The penicillin-resistant strains of *Staph. aureus* which are, today, such a menace in our hospitals, owe their resistance to the fact that they produce an enzyme, penicillinase, which inactivates penicillin, but are usually incapable of growing in an increased concentration of unchanged antibiotic. These staphylococci, apart from penicillinase production, resemble typical, fully virulent strains. On the other hand, when staphylococci are passaged in the presence of penicillin *in vitro*, they fairly quickly become drug tolerant; that is to say, they are able to grow in the presence of increased concentrations of penicillin but do not produce penicillinase. These staphylococci are only pale ghosts of the parent strains from which they were derived and have little or no capacity to produce coagulase or alpha-toxin or to cause disease in animals.

On the basis of these facts it has been suggested that a penicillin resistant to staphylococcal penicillinase would finally silence the adaptable staphylococcus. Before Celbenin is used too indiscriminately it seems of some importance to determine if this is indeed, the case, and to this end the present study was undertaken.

EXPERIMENTAL METHODS

Eighteen strains of *Staph. pyogenes* recently isolated from septic processes were selected for study. The antibiotic sensitivity patterns and phage types of the strains are given in Table I. Starting from single colonies the strains were passaged 45 to 50 times, at the rate of three to five times a week, on ditch plates containing increasing concentrations of Celbenin in the ditch. At intervals the strains were tested for sensitivity to benzyl penicillin and Celbenin by serial dilution in broth, with an inoculum consisting of 0·02 ml. of a 1/100 dilution of a six-hour shaken culture, which was equivalent to approximately 10^5 to 10^6 organisms.

Celbenin-resistant cultures isolated in this way were passaged in the absence of the antibiotic to see whether

[1] Methicillin is now the accepted name for the antibiotic first introduced by the Beecham Research Laboratories under the trade name Celbenin.

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the resistance was stable. Their cultural characteristics, sensitivity to other antibiotics, phage type, and coagulase and haemolysin activity were compared with those of the parent culture from which they were derived. For haemolysin production the cultures were grown on sloppy agar and incubated for 48 hours in an atmosphere containing an excess of CO_2; the cultures were then centrifuged and the supernatants tested for their capacity to haemolyse rabbit, sheep, and human red blood capsules.

Four of the Celbenin-resistant variants (two penicillin-sensitive and two penicillin-resistant) were tested for mouse virulence by the technique of Selbie and Simon (1952). An injection of 0·2 ml. of a six-hour shaken culture was injected into the right thigh; the diameter of the thigh was measured at daily intervals up to the seventh day and the degree of swelling estimated by subtracting the size of the opposite thigh.

RESULTS

DEGREE OF RESISTANCE The sensitivity to Celbenin of the parent strains and the cultures after 45 to 50 passages on Celbenin plates is shown in Table I. It will be seen that initially all 18 strains were inhibited by from 1·5 to 3·0 μg./ml. Celbenin. After passage in Celbenin the minimum inhibitory concentration was 100 or more for nine strains, while in three cases it was only 12. The remaining strains showed an intermediate degree of resistance.

The rate of increase in resistance of penicillin-sensitive and penicillinase-producing cultures is given in Table II. It will be seen that the penicillinase-producing cultures tended to become resistant more

TABLE I

CHARACTERISTICS OF PARENT STRAIN

Strain	Antibiotic Sensitivity						Phage Type	Sensitivity to Celbenin (M.I.C.) (μg./ml.)		
	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin		Parent Strain	After 45–50 Passages in Celbenin	
A Penicillin-destroying Strains										
1	R	S	S	S	S	S	6, 7, 47, 53, 54, 75	3·1	>100	
6	R	R	R	R	R	S	83	3·1	>100	
7	R	S	R	S	S	S	80, 81	3·1	50	
8	R	R	R	S	S	S	47	3·1	25	
14	R	S	S	S	S	S	6, 47, 53	1·5	>100	
15	R	S	S	S	S	S	52, 79	3·1	25	
16	R	S	S	S	S	S	52, 80+	3·1	50	
17	R	S	S	S	S	S	6, 47, 53, 77	3·1	50	
18	R	R	R	R	S	R	83	3·1	>100	
										Sensitivity of Celbenin R. variant to Penicillin (u./ml.)
B Penicillin-sensitive Strains										
2	S	S	S	S	S	S	3C, 55, 71	1·5	12	2·0
3	S	R	R	S	S	S	47, 53, 77, 83	1·5	12	1·0
4	S	S	S	S	S	S	52A, 79	1·5	12	1·0
5	S	S	S	S	S	S	6, 47, 53, 77, 83	1·5	50	100·0
9	S	S	S	S	S	S	3C, 55, 71	3·1	>100	2·0
10	S	S	S	S	S	S	6, 42E, 53, 54+	3·1	>100	6·25
11	S	S	S	S	S	S	80, 81	1·5	100	1·0
12	S	S	R	S	S	S	79, 53, 83+	3·1	>100	100·0
13	S	S	S	S	S	S	6, 47, 53	3·1	>100	100·0

TABLE II

DEGREE OF RESISTANCE TO CELBENIN

Concentration Celbenin (μg./ml.) Permitting Growth	Number of Passages									
	0		12		20–24		32		45–50	
	Penicillin Sensitive	Penicillin Resistant	Penicillin Sensitive	Penicillin Resistant	Penicillin Sensitive	Penicillin Resistant	Penicillin Sensitive	Penicillin Resistant	Penicillin Sensitive	Penicillin Resistant
100 or more	0	0	0	0	0	0	0	1	4	4
50	0	0	0	0	0	1	0	2	5	4
25	0	0	0	0	0	2	5	5	6	7
12·5	0	0	0	0	2	6	5	8	6	9
6·25	0	0	3	6	5	8	6	9	9	
3·1	0	0	6	9	9	9	9			
1·5	4	8	9							
0·75	9	9								

After passage in Celbenin of 18 strains *Staph. aureus*, (nine sensitive to penicillin (Pen. S) and nine penicillinase producing (Pen. R) . Figures represent the number of strains resistant to various concentrations.

quickly than the penicillin-sensitive strains. With two of the former (strain 1 and strain 6) the minimum inhibitory concentration of Celbenin was respectively 50 and 100 μg./ml. after 24 passages. After 45 to 50 passages, however, the same number of penicillin-sensitive and penicillin-resistant cultures showed a gross increase in resistance to Celbenin.

CELBENIN DESTRUCTION Broth cultures of strains resistant to more than 100 μg./ml. were incubated with equal quantities of a solution containing 250 μg./ml. Celbenin for three hours and the active Celbenin in the mixture was subsequently estimated by the cup-plate method. In no case was there any inactivation of Celbenin.

ASSOCIATED INCREASE IN RESISTANCE TO BENZYL PENICILLIN Initially all penicillin-sensitive strains were inhibited by 0·06 unit/ml. benzyl penicillin. As will be seen from Tables I and III, after passage in

TABLE III

DEGREE OF RESISTANCE TO BENZYL PENICILLIN AFTER PASSAGE IN CELBENIN OF NINE PENICILLIN-SENSITIVE STRAINS *STAPH. AUREUS*

Concentration of Penicillin (u./ml.) Permitting Growth	*No. of Passages*		
	20-24	32	45-50
50 or more	—	—	3
2	0	3	4
1	3	5	6
0·5	5	9	9
0·25	8	9	9
No. of strains inhibited by 0·25 u./ml. or less	1	0	0

Figures represent the number of strains resistant to various concentrations.
All strains were initially inhibited by 0·06 u./ml.

Celbenin all showed a considerable increase in resistance to benzyl penicillin. This increase was invariably greater than the increase in resistance to Celbenin but strains showing the greatest increase in resistance to one did not necessarily show the greatest increase in resistance to the other antibiotic. A strain showing a gross change in sensitivity to both antibiotics is shown in Fig. 1a.

The penicillin-destroying cultures 1 and 6 were tested for their sensitivity to penicillin using a small (approximately 500 cells) and large (approximately 1 million cells) inoculum. They retained their full capacity to produce penicillinase and in addition they had become penicillin tolerant, so that the resistance to penicillin was similar whether a small or large inoculum was tested. This was clearly demonstrated when the cultures were plated on penicillin ditch plates and examples are shown in Fig. 1b, 1c, and 1d. It will be seen that the resistant organisms in the culture obtained from strain 7 (Fig. 1d) are small colony variants whereas with strain 1 (Fig. 1b) and strain 6 (Fig. 1c) the resistant variant more closely resembles the parent strain.

CULTURAL CHARACTERISTICS AND STABILITY OF CELBENIN-RESISTANT VARIANTS All 13 strains resistant to 25 μg./ml. or more Celbenin were compared with their parent strains in relation to cultural characteristics and were tested for the stability of their resistance to Celbenin by serial passage in nutrient agar in the absence of the antibiotic. In these respects there was a sharp difference between the cultures derived from penicillin-sensitive strains and those derived from penicillin-destroying strains.

All the six penicillin-sensitive, Celbenin-resistant variants multiplied less rapidly in artificial medium than did their parent strains and when placed on nutrient agar gave rise to colonies which varied very much in size but most of which were smaller than a typical staphylococcal colony. When plated on Celbenin ditch plates the colonies growing in the neighbourhood of the Celbenin were always small, whereas away from the ditch colonies sometimes resembled those of the parent culture. These Celbenin-resistant variants tended to be very unstable and after only six passages in the absence of Celbenin often reverted almost completely to Celbenin-sensitive organisms. Typical examples are given in Figs. 2 to 4.

On the other hand the Celbenin-resistant variants isolated from penicillin-destroying strains, with one exception (strain 7, Fig. 1d), had cultural characteristics similar to those of the parent strains from which they were derived and they retained full resistance to Celbenin after 12 passages in the absence of the antibiotic. On first isolation from ditch plates the Celbenin-resistant variants showed a slightly longer lag phase in artificial culture media and gave rise to colonies slightly smaller than those of the parent strain, but these differences became less marked after passage in the absence of Celbenin although resistance to Celbenin was maintained. A typical example is given in Fig. 5.

PHAGE TYPE OF CELBENIN-RESISTANT VARIANTS The cultures obtained from all 18 strains after 45 to 50 passages on celbenin ditch plates were all phage-typed together with their parent strains. In all cases the cultures retained their phage-sensitivity and were of the same type as the parent strain.

SENSITIVITY TO OTHER ANTIBIOTICS OF CELBENIN-RESISTANT VARIANTS The sensitivity of all 18 Celbenin-resistant cultures to streptomycin, tetracycline, erythromycin, and novobiocin was similar

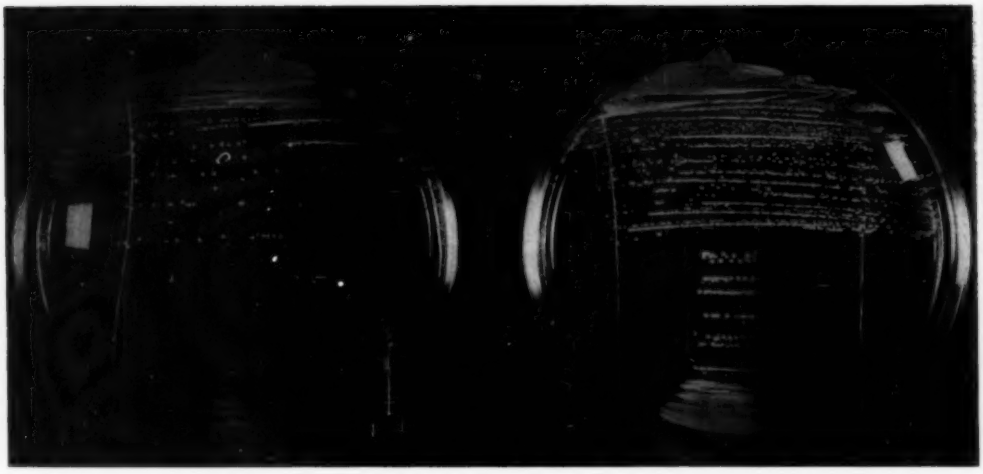

FIG. 1a

FIG. 1b

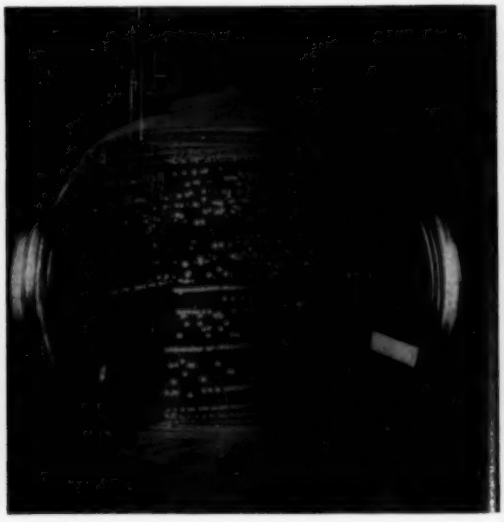

FIG. 1c

FIG. 1d

FIG. 1. *Celbenin-resistant variants (upper half of plate) and parent cultures (lower half of plate) plated on double ditc plates. Ditch with white disc contains Celbenin (50 μg. ml.) and opposite ditch benzyl penicillin (10 unit/ml.)*

FIGS. 2-5. *Typical cultures plated on ditch plates with 250 μg./ml. Celbenin in ditch. Lower half of each plate seede with parent culture; upper half of left plate seeded with culture immediately after 45 passages in Celbenin; upper half o right plate seeded with resistant variant after six passages on Celbenin-free medium.*

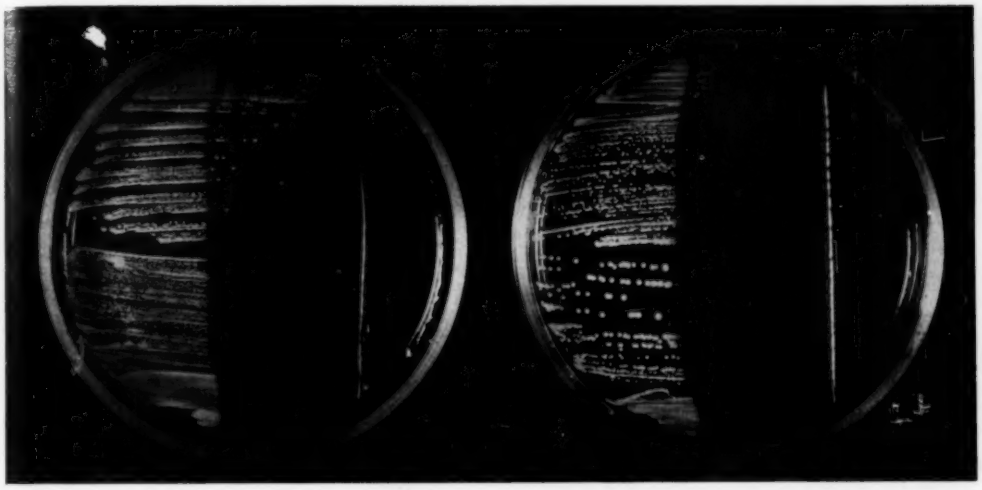

FIG. 2. *Strain 10.*

FIG. 3. *Strain 12.*

to that of the parent strains. Both the strains (6 and 13), which were initially resistant to chloramphenicol, became sensitive to this antibiotic after passage in Celbenin.

COAGULASE ACTIVITY Young broth cultures of all the Celbenin-resistant strains clotted citrated plasma in 30 to 60 minutes and there was no significant difference in the rate of clotting by parent or passaged cultures, provided the cultures were first adjusted to the same opacity.

HAEMOLYSIN PRODUCTION All the eight cultures resistant to 100 μg./ml. or more Celbenin were tested

FIG. 4. *Strain 13.*

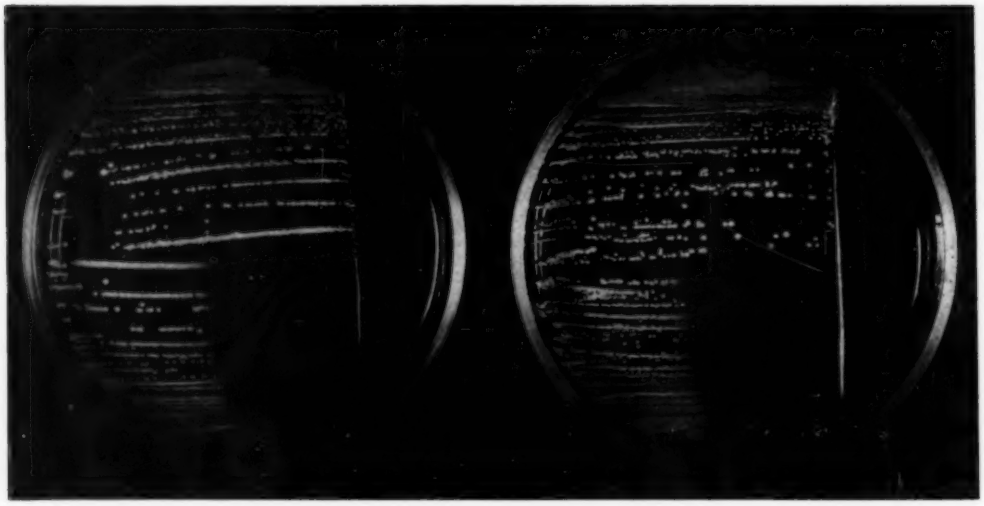

FIG. 5. *Strain 6.*

for haemolytic activity against rabbit, sheep, and human red blood cells. Two cultures showing an intermediate degree of resistance were tested with sheep cells only. The results are given in Table IV. In most cases the activities of the parent and the Celbenin-resistant variant were similar, but with strain 18 and strain 13 the Celbenin-resistant culture had a significantly lower activity than the paren culture.

MOUSE VIRULENCE The virulence of five Celbenin resistant cultures was compared with that of thei parent strains by measuring the amount of swellin in the thighs of mice after intramuscular injection

TABLE IV

HAEMOLYSIN PRODUCTION

Strain		Stability of Resistance to Celbenin	Haemolytic Titre			Mouse Virulence Measured as Average Diameter (mm.) of Swelling		
			Rabbit Red Cells	Sheep Red Cells	Human Red Cells	3rd day	5th day	7th day
1	Parent	—	1/160	1/20	1/8	5·7	8·0	9·0
	Celbenin R	Stable	1/80	1/10	1/8	7·5	9·0	9·0
6	Parent	—	1/320	1/40	1/8	4·0	4·0	5·0
	Celbenin R	Stable	1/320	1/20	1/4	4·3	4·3	6·3
7	Parent	—		1/32		5·3	4·3	4·6
	Celbenin R	Unstable		1/32		5·3	4·7	5·3
14	Parent	—	1/320	<1/5	1/4			
	Celbenin R	Stable	1/320	<1/5	<1/2			
18	Parent	—	1/320	1/10	1/8			
	Celbenin R	Stable	1/80	<1/5	<1/2			
5	Parent	—		1/16		3·0	4·0	4·0
	Celbenin R	Moderate		1/16		2·0	2·7	2·7
9	Parent	—	1/320	1/20	1/8			
	Celbenin R	Unstable	1/320	1/10	1/4			
10	Parent	—	1/10	<1/5	<1/2			
	Celbenin R	Very unstable	1/10	<1/5	<1/2			
12	Parent	—	1/640	1/40	1/8			
	Celbenin R	Very unstable	1/640	1/40	1/8			
13	Parent	—	1/320	1/10	1/4	3·7	4·0	5·3
	Celbenin R	Moderate	1/40	1/5	<1/2	1·0	1·0	1·0

Groups of three mice were used for each culture. The average diameter of swelling on the third, fifth, and seventh days is shown in Table IV. With the penicillin-destroying strains 1, 6, and 7 the virulence of the Celbenin-resistant strain was at least as high as that of the parent culture.

The two penicillin-sensitive strains tested, strains 5 and 13, were selected because the Celbenin-resistant variant was rather more stable in its resistance than was the case with the other strains. It will be seen that the Celbenin-resistant variant of strain 5 caused less swelling than the parent culture and that the passaged variant of strain 13 was almost avirulent.

PASSAGE IN BENZYL PENICILLIN The parent cultures of the penicillin-destroying strains 1 and 6 and the penicillin-sensitive strains 5 and 12 were serially passaged on penicillin ditch plates. After 45 passages strains 1 and 6 showed respectively a two- and four-fold increase in resistance to celbenin.

Both the penicillin-sensitive strains showed a 200-fold increase in resistance to benzyl penicillin after 45 passages, but only a four-fold increase in resistance to Celbenin.

NATURALLY OCCURRING CELBENIN-RESISTANT STRAINS For the last nine months all strains of *Staph. pyogenes* isolated in the laboratory of this hospital have been tested for sensitivity to Celbenin by a ditch-plate method. During this period more than 500 strains from infective processes have been tested and only one strain was found to be resistant to Celbenin. This strain came from a bedsore on a child from St. Mary's Cray who had been treated with penicillin and streptomycin, but had never had Celbenin. The phage type and sensitivity to other antibiotics of this strain (strain C.R.1) are given in Table V.

Two other naturally occurring Celbenin-resistant staphylococci have been kindly sent to me by Dr. Riddell (strain C.R.2) and Dr. Patricia Jevons (strain C.R.3). The former was isolated from a patient who lived in Farnborough and the latter was isolated from several patients in a hospital near

TABLE V

NATURALLY OCCURRING CELBENIN-RESISTANT STRAINS

Strain	Source	Antibiotic Sensitivity						Phage Type
		Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	Novobiocin	
C.R. 1	General hospital	R	R	R	S	R	S	53, 54, 75, 77
C.R. 2	General hospital	R	R	R	S	S	S	7, 47, 53, 75, 77
C.R. 3	Central Public Health Laboratory	R	R	R	S	S	S	7, 47, 53, 54, 75, 77

Guildford (see Jevons, 1961). None of these patients had been treated with Celbenin. The sensitivity to other antibiotics and phage type of these strains are given in Table V. It will be seen that all three strains belonged to phage group 3 and were of closely related, if not the same, phage types.

Degree of Resistance to Celbenin All three strains behaved in a similar way in the presence of Celbenin. When sensitivity tests were carried out in fluid media with a small or moderate-sized inoculum and read after 24 hours' incubation the strains showed only a moderate degree of resistance and were inhibited by from 12·5 to 25 μg./ml. Celbenin. On solid medium only a small number of colonies grew in the presence of a high concentration of Celbenin, and moreover these colonies were not visible after overnight incubation and even after 48 hours' incubation were much smaller than typical staphylococcal colonies.

Passage in Celbenin After five passages on Celbenin ditch plates all the strains grew in 250 to 500 μg./ml. of Celbenin after overnight incubation. On solid media containing 5 μg./ml. colonies were very variable in size, ranging from typical staphylococcal colonies to very tiny ones. On plates containing 25 μg./ml. or more all colonies were small.

Stability of Resistance After six passages on nutrient agar in the absence of Celbenin all three strains had a reaction to Celbenin similar to that of the original strains.

It is thus apparent that these three naturally resistant organisms on first isolation consisted predominantly of cells with only a moderate degree of resistance to Celbenin. After a few passages in the presence of the antibiotic the highly resistant organisms were selected at the expense of the more sensitive cells. But although the highly resistant cells were capable of growth in the presence of very large concentrations of Celbenin, their degree of growth and colony size was much less when the Celbenin concentration was 25 μg./ml. or more than with lower concentrations.

DISCUSSION

After repeated passage on Celbenin ditch plates all of 18 strains of *Staph. pyogenes* showed an increase in resistance to Celbenin and eight of the strains were capable of growing in 100 or more μg./ml. Celbenin. Resistance was of the drug-tolerant type, and none of the resistant strains inactivated Celbenin. There was an associated increase in tolerance to benzyl penicillin.

As is well known, staphylococci readily develop resistance of this type *in vitro* to benzyl penicillin. There is, however, a very remarkable difference between *in vitro* induced benzyl-penicillin-resistant staphylococci and the Celbenin-resistant organisms isolated from penicillinase-producing staphylococci reported in this communication.

Thus staphylococci trained to be penicillin G-tolerant, although of several different types (see Barber, 1953), always grow much less luxuriantly than typical staphylococci and are of reduced coagulase and alpha-toxin activity and virulence (Rake, McKee, Hamre, and Houck, 1944; Spink, Ferris, and Vivino, 1944; Blair, Carr, and Buchman, 1946). It is probably on account of these facts that they are rarely encountered in clinical practice. These organisms are also extremely unstable and tend to lose their resistance to penicillin rapidly on subculture in the absence of antibiotic.

Four of the penicillin-destroying strains used in the present investigation have yielded stable Celbenin-tolerant cultures which are resistant to 100 or more μg./ml. Celbenin. All four resembled the parent strains in cultural characteristics and coagulase production, and in three cases the haemolytic activity was similar. Two of the strains were tested for mouse virulence by injection into the thigh and the Celbenin-resistant cultures were shown to be as virulent as the parent strains.

Four of the penicillin-sensitive strains yielded cultures with a similar degree of tolerance to Celbenin, but in other respects were in sharp contrast to the cultures isolated from penicillin-destroying strains. Thus the cultures were unstable and tended to become Celbenin-sensitive in the absence of the antibiotic. The resistant cultures grew much less luxuriantly than their parent strains. On subculture in the absence of Celbenin these cultures rapidly reverted to cultures resembling the parent strain in sensitivity to Celbenin and colonial appearances.

No explanation has been found for the difference in the Celbenin-resistant organisms isolated from penicillin-destroying and penicillin-sensitive staphylococci. In neither case do the resistant organisms inactivate Celbenin. But, as pointed out by Hayes (1957), two mutations are probably involved in the production of penicillinase-producing staphylococci. Only mutation 2 is concerned with the production of inducible penicillinase, but this mutation cannot express itself in the absence of mutation 1 which results in cells able constitutively to produce very small amounts of the enzyme. Celbenin is not completely resistant to staphylococcal penicillinase and it is possible that mutation 1 would render staphylococci better able to deal with Celbenin at the cellular level.

Naturally occurring Celbenin-resistant strains of *Staph. pyogenes* are not common. Jevons (1961), of the Central Public Health Laboratory, only en-

countered one (the strain referred to in this paper as C.R.3) in a study of 4,340 strains from routine material from hospitals. This and the two other strains described here grow very poorly in the presence of more than 12 to 25 μg./ml. Celbenin and may, therefore, lose much of their virulence in the presence of the antibiotic.

Since naturally occurring Celbenin-resistant organisms are not only rare but of doubtful clinical significance, it has been suggested (Rolinson, 1961; Knox, 1961) that Celbenin-resistant staphylococci are not likely to be of clinical importance. This may be the case. Certainly staphylococci cannot develop resistance to Celbenin at the rate they can do to erythromycin and novobiocin, and it is unlikely that a gross increase in resistance to Celbenin will occur during the treatment of a single patient.

But two at least of the Celbenin-tolerant cultures isolated in this investigation from penicillinase-producing staphylococci have apparently retained their virulence. Such organisms might arise by passage in patients treated with Celbenin, if the antibiotic be used extensively in wards where cross-infection is occurring. These two Celbenin-tolerant cultures retained their full capacity to produce penicillinase, unlike the strains described by Fairbrother and Taylor (1961).

Clearly Celbenin-resistant staphylococci are a potential danger. It should be remembered that staphylococci do not readily become resistant to the tetracyclines and early reports on chlortetracycline suggested that tetracycline-resistant staphylococci were unlikely to be of clinical importance (Paine, Collins, and Finland, 1948; Finland, Collins, and Paine, 1948). It is unwise to assume that the staphylococcus has met its match. In the meantime Celbenin should be used with discrimination, in which case it will remain an invaluable weapon for the treatment of staphylococcal infection.

It is a pleasure to thank Dr. M. P. Jevons, of the Central Public Health Laboratory, and Dr. R. W. Riddell, of the Brompton Hospital, for sending me naturally occurring Celbenin-resistant strains and supplying me with full details of these strains including the phage-types. I am also very grateful to the directors of Beecham Research Laboratories for a free supply of Celbenin.

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Coccidiosis in guinea-pigs

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SYNOPSIS The attention of laboratory workers is drawn to the possibility of coccidiosis as a cause of death in guinea-pigs. The purchase of a number of guinea-pigs infected with this protozoon was followed by 12 deaths when these animals were injected with material for diagnostic purposes. No deaths occurred in the laboratory stock herd, as these were kept separate from the newcomers and were not infected.

The life history of the parasite is described, together with the post-mortem findings in our series of animals.

MATERIAL AND METHODS

Because of an increase in the number of requests for biological examinations in April 1960, a stock of guinea-pigs was purchased and used without a preliminary quarantine period. These animals received an intramuscular injection into the right thigh of material such as uterine curettings, urine, or milk, and of those injected 12 subsequently died. All the deaths occurred among the imported animals and followed a brief illness in which diarrhoea was the principal symptom. The imported guinea-pigs were kept separate from the laboratory stock and no deaths or illness occurred in the latter group.

POST-MORTEM EXAMINATION

Post-mortem examination, together with bacteriological investigations, were performed on all guinea-pigs dying and, in addition, material was frozen and kept in case virological examination might be required.

Direct wet preparations of faeces were examined for parasites and cysts were sought for after concentration by the salt flotation method.

Material for histological examination was fixed in formalin and sections were stained by haematoxylin and eosin. A comprehensive histological examination was performed on only three of the guinea-pigs.

RESULTS

GROSS APPEARANCE OF ORGANS The visceral peritoneum was congested and there was bloodstained fluid in the peritoneal cavity. White patches on the outer surface of the colon were taken to be patches of lymphoid tissue.

BACTERIOLOGY Bacteriological examination was negative and in view of the findings described below virological studies were not undertaken.

HISTOLOGY The only abnormal findings outside the intestional tract were areas of pulmonary collapse.

The visceral peritoneum and mesentery in relation to the gut showed a non-specific inflammatory infiltration consisting of a mixture of polymorphs and chronic inflammatory cells.

The whitish patches on the colon were areas of lymphoid tissue, some of which showed ulceration with a surrounding inflammatory reaction. Ulceration appeared particularly liable to occur within small mucosal diverticuli and, in sections not passing through the opening of the diverticulum, produced the appearance of abscess cavities lying within the submucosa. The mucosa of the colon near these lesions contained enormous numbers of cysts measuring 18 × 15 μ which were indentified as the oocysts of the protozoal parasite *Eimeria caviae*. The cysts had a well-defined capsule and contained a mass of granular cytoplasm.

In one case no ulceration was found and only a few degenerated oocysts were present in the colon, together with a non-specific enteritis affecting most of the small intestine.

In the infected animals, three further stages in the life cycle of *Eimeria caviae* were found in the deeper parts of the mucosa: (1) the schizont, (2) the macrogametocyte, and (3) the microgametocyte.

THE LIFE CYCLE OF THE ORGANISM

Maturation of oocysts was studied by emulsifying a small portion of faeces in 5% sodium dichromate

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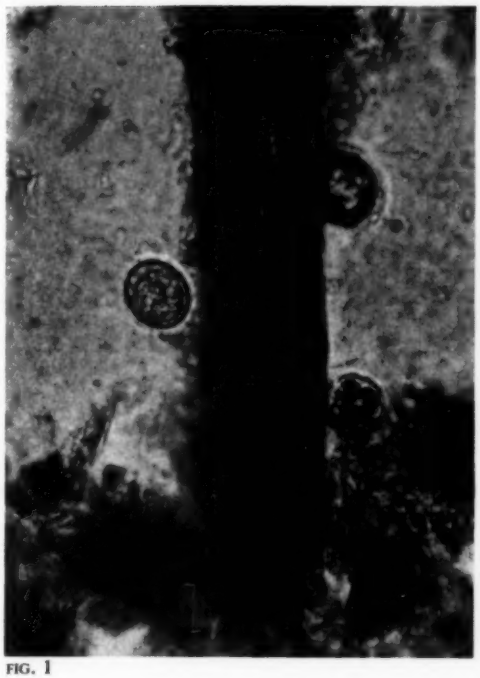

FIG. 1

FIG. 2

FIG. 1. *Three oocysts, one of which is in the process of division into four sporocysts.*

FIG. 2. *A section of large intestine containing three parasites. The centre one, with a prominent nucleolus, is a macrogametocyte* (♀). *Near to this is a microgametocyte* (♂) *showing nuclear division into microgametes.*

and placing it in a Petri dish at room temperature for a period of four to five days. At the end of this time the oocysts showed division into four distinct masses (Fig. 1) representing the four sporocysts characteristic of the genus *Eimeria*. In the natural cycle this mature oocyst is ingested and the sporocysts each release a pair of sporozoites which escape to infect the epithelial cells of the large intestine.

The first, asexual, stage of reproduction, that of schizogony, then takes place and is followed by the liberation of merozoites capable of entering more epithelial cells and repeating the asexual cycle.

The asexual forms appear as inclusions within the cytoplasm of epithelial cells, young individuals being smaller than the cell nucleus. Older parasites are larger and show several nuclei. At a later stage a sexual process occurs, microgametocytes (male) and macrogametocytes (female) being produced (Fig. 2).

The macrogametocyte is a prominent cell with a round nucleus containing a small chromatin condensation. Within the cytoplasm are numerous coarse granules.

The microgametocyte is of similar size but instead of a single discrete nucleus it contains a large number of elongated or comma-shaped nuclear bodies which represent the microgametes. Rupture of the microgametocyte releases the microgametes which, by means of their flagellae, swim to the macrogametocytes which they fertilize. After fertilization the cytoplasmic granules of the macrogametocytes fuse to produce the oocyst capsule. The oocyst is passed in the faeces and matures outside the body of the host. On being ingested by a suitable host the sporozoites are liberated and the asexual cycle is repeated.

DISCUSSION

Coccidiosis is due to infection of the alimentary tract with protozoa of the genus *Eimeria*, class Sporozoa, all members of which are obligatory intracellular parasites. Within the genus there is a large number of species all showing a high degree of host specificity. Practically all domestic animals are potential hosts for *Eimeriae* but coccidiosis is best

known as a disease of poultry and rabbits in which it may reach epidemic proportions resulting in serious economic loss. Man is not a host for any species of *Eimeria* although the closely related *Isospora* occasionally causes a dysentery-like illness. Those engaged in medical laboratory work are therefore apt to be unfamiliar with the disease.

Infection of guinea-pigs with *Eimeria caviae*, whose full life cycle was first described by Sheather (1924), was at one time considered to be extremely common but of little consequence. This view, endorsed by Henry (1932) and Lapage (1940), is also expressed in textbooks of protozoology such as that of Wenyon (1926). It is possible that the care taken in the maintenance of laboratory guinea-pigs in recent years has resulted in a decrease in the incidence of infection. Under experimental conditions, however, healthy guinea-pigs can be infected by feeding with washed suspensions of cysts (Henry, 1932). If the dose is large, clinical illness may follow with diarrhoea and eventually death. In the natural course of events death does not usually ensue and it would appear that it can do so only when infection is heavy and is combined with other factors which upset the balance between the organism and its host.

The recent importation into this laboratory of a number of infected guinea-pigs appears to have provided the necessary upset in equilibrium. In this instance, transportation, the introduction to new surroundings, different food, and finally the intramuscular injection of clinical material for diagnostic purposes, may have had some bearing on the appearance of intestinal ulceration with secondary infection, diarrhoea, and eventual death.

Once the diagnosis was established we were able to confirm that the imported guinea-pigs were infected and although these had been kept separate from our breeding herds we treated all guinea-pigs by adding sulphamezathine to their drinking water. An interrupted course of treatment was used in the form of three days' treatment and two days' rest, repeated to a total of nine days' treatment, and proved successful.

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Rapid isolation of salmonellae from faeces

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SYNOPSIS Factors and techniques leading to the more rapid isolation of salmonellae from faeces that are negative by direct plating were examined. The aim was to increase the number of specimens on which positive reports might be given within 24 hours of receipt. Salmonellae were isolated from 279 of the specimens studied.

Brilliant green MacConkey agar was found to be the most satisfactory solid selective medium both for direct plating and as a subculture medium, giving large characteristic colonies after 24 hours' incubation. Selenite F medium, inoculated with undiluted faeces, incubated at 43°C., and subcultured after six hours' incubation on to brilliant green MacConkey agar, was the most successful rapid method of enrichment, though the results were considerably inferior to those obtained after 24 hours' incubation. Seventy-six of the positive specimens examined were negative by direct plating on three different selective media; 24 (31·6%) of these were positive on subculture from selenite F incubated for six hours at 43°C. and 21 (27·6%) when incubation was at 37°C. The use of six-hour subcultures from 43°C. selenite F as a supplement to direct plating added significantly to the number of positive results. Other techniques that were tried but found to be of no practical value were dilution of the faeces before inoculation on solid and fluid media, and replica plating from one solid medium to another after a few hours' incubation.

The rapid identification of excreters of even small numbers of salmonellae is of importance when an outbreak of food poisoning occurs in a hospital or residential institution, or when the staff of a food establishment is involved. The usual routine methods for the isolation of salmonellae from faeces consist of direct inoculation of one or more selective solid culture media, together with inoculation of one or more fluid enrichment media which, after overnight incubation at 37°C., are subcultured on to selective agars. Salmonella colonies are not found on direct culture plates of a number of specimens subsequently found to be positive, and these specimens cannot be reported on until subcultures from fluid media are examined 40 or more hours after the specimens have been received.

This paper records the results of an investigation of some techniques that might facilitate the isolation of salmonellae within 24 hours from specimens that are negative by direct plating. Three solid selective media were evaluated with regard to the rapid growth of characteristic colonies. Rapid enrichment was attempted on solid media by a replica plating technique, and with fluid media by subculture after only six hours' incubation.

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MATERIALS

The faeces specimens were received during a period of one year from patients suspected of excreting salmonellae other than *S. typhi* and *S. paratyphi*. Most of the specimens were from untreated convalescent excreters; those known to be from patients being treated with antibiotics were omitted. *S. typhimurium* was isolated from 246 (88·2%) of the 279 positive stools, *S. enteritidis*, *S. newport*, and *S. thompson* being the other serotypes found.

THE USE OF SOLID MEDIA

CHOICE OF SOLID SELECTIVE MEDIUM AND EFFECT OF DILUTING THE INOCULUM Three selective media were chosen, *viz.*, deoxycholate-citrate agar (Leifson, 1935; Hynes, 1942), Wilson and Blair's bismuth sulphite agar (Wilson and Blair, 1927; De Loureiro, 1942), and MacConkey agar to which 1 : 25,000 brilliant green had been added. Whole plates of media were inoculated with faeces and further plates with a loopful of a 1 : 1,000 dilution of faeces in quarter-strength Ringer's solution. The plates were examined after 24 hours' incubation at 37°C. and suspicious colonies were tested by slide agglutination with diagnostic sera and by the usual biochemical tests. For comparative purposes selenite F medium (Leifson, 1936) was inoculated with faeces and subcultured after 24 hours' incubation at 37°C.

TABLE I

NUMBER OF ISOLATIONS OF SALMONELLAE WHEN SOLID AND FLUID MEDIA WERE INOCULATED WITH DILUTED AND UNDILUTED FAECES

	Direct Examination			*Selenite F (24 hr.)*
	BGM	*DC*	*WB*	
Undiluted faeces	189	146	94	266
1 : 1,000 faeces	135	98	56	205

BGM = brilliant green MacConkey agar; DC = deoxycholate citrate agar; WB = Wilson and Blair's medium. The selenite F medium was subcultured onto brilliant green MacConkey agar after 24 hours' incubation at 37°C. The solid media were examined after 24 hours' incubation.

The results of the examination of 279 positive specimens are presented in Table I. Brilliant green MacConkey agar was the most successful of the three media. In most instances the colonies on the brilliant green MacConkey agar were more than twice the diameter of the corresponding colonies of the pathogens on the other two media. The medium was prepared by the addition of brilliant green in a concentration of 1 : 25,000 to ordinary MacConkey agar including neutral red. The amount of brilliant green required may vary with the particular brand or batch of dye and sodium taurocholate, but is readily determined by testing plates containing concentrations of dye in the range 1 : 20,000 to 1 : 35,000. Judex JTS brilliant green[1] has been found to be very satisfactory. Specimens found positive by direct plating on one or more media after inoculation with undiluted faeces totalled 203; inoculation of diluted faeces resulted in 150 isolations. Dilution of the specimens had no value in aiding the rapid isolation of salmonellae by direct plating on any of the three media.

REPLICA PLATING AND ENRICHMENT ON SOLID MEDIA The technique used was that of Lederberg and Lederberg (1952). A plate of medium was inoculated with faeces and incubated for two to five hours at 37°C. Bacteria from the micro-colonies that resulted were then transferred to a velvet surface by pressing the inoculated surface of the agar on to the velvet. The velvet was secured to one end of a cylindrical wooden block of slightly smaller diameter than a Petri dish. Replicate inoculations from the velvet surface on to other selective and non-selective media were then made by pressing on further plates. The replicate plates were incubated at 37°C. for 18 hours and then examined as described in the previous section.

Various combinations of the three selective media previously described and MacConkey agar were used for primary and replicate platings, but none of the combinations was of value in the enrichment of small numbers of salmonellae present in specimens of faeces. It was sometimes difficult to recover salmonellae by these methods from faecal specimens that were found to be positive by direct plating.

[1]The General Chemical and Pharmaceutical Co Ltd., Sudbury Middlesex.

THE USE OF EARLY SUBCULTURE FROM FLUID MEDIA

Tubes of selenite F medium (Leifson, 1936) and nutrient broth were inoculated with approximately 0·1 g. faeces or 1 ml. of a 1 : 1,000 dilution of faeces. The tubes were incubated in a water-bath at either 37°C. or 43°C. and subcultured on to brilliant green MacConkey agar plates after six hours' incubation. The plates were incubated for 18 hours at 37°C., then examined as already described. For comparison, the faeces samples were also inoculated directly and in a 1 : 1,000 dilution on to the three selective agars previously used, and the selenite F incubated at 37°C. was subcultured after 24 hours' incubation. Salmonellae were isolated from 279 of the specimens. The results of most interest were those of the 76 positive specimens that were negative on the three selective media inoculated with undiluted faeces; they are presented in Table II. Selenite F inoculated with undiluted faeces and incubated at 43°C. gave the greatest number of positive results of the rapid methods; 24 isolations were made,

TABLE II

RESULTS OF EXAMINATION BY VARIOUS ENRICHMENT PROCEDURES OF 76 POSITIVE FAECAL SPECIMENS FOUND NEGATIVE FOR SALMONELLAE BY PLATING AN UNDILUTED INOCULUM ON THREE SELECTIVE AGARS

	Six-hour Subculture			*24-hour Subculture*
	Selenite 37°C.	*Selenite 43°C.*	*Broth 43°C.*	*Selenite 37°C.*
Undiluted faeces	21	24	19	68
1 : 1,000 faeces	7	6	—	32

All subcultures were on to brilliant green MacConkey agar.

which represented 31·6% of the specimens that were negative by direct plating but from which salmonellae were finally isolated. These, however, were far below the number (68) isolated from selenite cultures incubated for 24 hours at 37°C. Incubation of selenite for six hours at 37°C. gave 21 positives (27·6%) and nutrient broth at 43°C. yielded 19 (25·0%). Dilution of the faeces before inoculation of selenite was not helpful at either temperature of incubation.

The results of the 279 positive specimens were analysed to establish the value of six-hour subculture from selenite F incubated at 43°C. as a supplement to direct plating on to three selective media. Triple direct plating gave 203 positive results 24 hours after receipt of the specimens, whereas the additional use of 43°C. six-hour selenite enrichment increased the number found positive at this time to 227; this increase is significant by the χ^2 test ($\chi^2 = 5{\cdot}79$; $P < 0{\cdot}02$). A more significant increase was demonstrated when the short enrichment was used as a supplement to direct plating on to a single medium. One hundred and eighty-nine specimens of faeces were positive at 24 hours by the most successful single plating medium (brilliant green MacConkey agar) and this figure was increased to 223 by the additional use of six-hour subculture from selenite F at 43°C. ($\chi^2 = 10{\cdot}69$; $P < 0{\cdot}01$)

DISCUSSION

The value of brilliant green MacConkey agar for the isolation of salmonellae was demonstrated by Harvey (1956). The present investigation confirms this finding and indicates the rapidity with which characteristic salmonella colonies develop on the medium; for this reason it is of particular value for six-hour subcultures from fluid media which are, of necessity, made late in the day. The possibility that use could be made of the differing action of various selective agars led to the experiments making use of the replica plating technique of Lederberg and Lederberg (1952). Possibly the inhibitory action of the selective media allowed too few bacteria to be transferred to the replica plates for the method to be of value.

There are few reports on the value of early subculture from enrichment media. Boecker and Silberstein (1932), Boecker (1935), Waldhecker (1935), and Fry (quoted by Hobbs and Allison, 1945) found early subculture from tetrathionate media of value. H. W. Smith (1952) could not recover salmonellae before the fifteenth hour of incubation from selenite and tetrathionate broths that had been inoculated with artifically-infected faeces. H. G. Smith (1959), however, working with pure cultures, considered the selective action of selenite broth to occur quite early in the growth period. Graphs presented by Leifson (1936) indicated that typhoid bacilli outnumbered coli more quickly in selenite F when a 1 : 1,000 dilution of faeces was inoculated than when undiluted faeces were used. Diluted faeces have been used with success for inoculating selective agars with specimens from chronic enteric carriers (Thomson, 1954), but had no advantage over undiluted faeces when used with either solid or fluid media in the present study. The report by Harvey and Thomson (1953) that incubation of selenite broth at 43°C. facilitated the isolation of *S. paratyphi B* from faeces suggested the use of this temperature for rapid enrichment in selenite. As selenite F does not support the growth of salmonellae as well as nutrient broth (Banwart and Ayres, 1953), and Thomson (1955) noted that nutrient broth was of some value for the isolation of *S. typhimurium* from faeces, a trial of nutrient broth incubated at 43°C. for six hours was also included.

Duplication of media inoculated with specimens will always increase the number of isolations of pathogens, but the degree of increase will vary with the detail of the method used. In the present investigation six-hour subcultures from selenite F inoculated with undiluted faeces and incubated at 43°C. gave the greatest increase in isolations of salmonellae within 24 hours. Incubation at 37°C. gave slightly fewer positive results than at 43°C. but some laboratories might find the former temperature more convenient. Most of the specimens examined were from convalescent cases because of the difficulty of obtaining a sufficient number of scanty positives from contacts, but there was no indication that these gave different results from specimens from contacts. The impression was gained during the serial examinations of faeces from convalescent cases that, after direct plating had become negative, six-hour enrichment continued to be positive for a few days until finally the pathogens could be found on 24-hour subculture plates only. It is not known whether similar results would have been obtained with specimens containing other salmonella serotypes, because the degree of enrichment occurring in selenite broth varies from species to species (H. W. Smith, 1952; Rappaport, Konforti, and Navon, 1956). *S. typhimurium*, which predominated in this study, has been the salmonella found most frequently in faeces in Great Britain for many years, and the other species isolated were among the six isolated most frequently from human excreters in 1958 (Report of Public Health Laboratory Service, 1959). There are certain circumstances when the speedy detection of persons excreting small numbers of salmonellae is important; the more successful techniques described in this paper might then prove of value when used as a supplement to direct plating.

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The estimation of magnesium in small biological samples by flame spectrophotometry

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SYNOPSIS Studies of human magnesium metabolism in this laboratory called for a simple and reliable method of estimating the magnesium content of large numbers of small biological specimens (Montgomery, 1960). It has been found that a technique involving the Unicam S.P. 900 flame spectrophotometer fulfils this need.

Estimations have been made on serum, red cells, urine, and acid digests of muscle and faeces. A standard instrument was used, burning acetylene and air, with a new standard atomiser containing a metal jet. Readings were made at the atomic line of 285·2 mμ.

FLAME CHARACTERISTICS

The difficulties and complications involved in studying the magnesium emission, and the disadvantages of using the oxide line at 371 mμ, have been discussed by Teloh (1958) and by Van Fossan, Baird, and Tekell (1959). In general, magnesium is liable to interference by sodium and potassium and to a lesser extent by calcium and phosphate in biological fluids. Practical problems are presented by the high concentration of sodium in serum and of potassium in intracellular fluid. At the magnesium atomic line, part of the sodium effect is due to the presence of a sodium line at 285·3 mμ, which should be readily corrected by the use of a sodium blank. There is also, however, a more general effect of mutual interference by extraneous cations; from analysis with a multichannel instrument, Margoshes and Vallee (1956) concluded that this is due to variation of heterochromatic background radiation in the presence of the specific flame, and not to intrinsic alteration of its monochromatic emission.

In the S.P. 900 instrument, spraying 1 : 10 dilutions of serum and 1 : 50 extracts of tissue, these effects can be corrected by the use of blanks and standards with the appropriate electrolyte concentrations. The background interference curves of both sodium and potassium in increasing concentrations with constant magnesium were found to approach the horizontal at concentrations above 10 mEq. per litre of sodium and 1 mEq. per litre of potassium. In other words, using the appropriate 'normal' blanks, no significant variation was found in the readings of a magnesium solution of 0·2 mEq. per litre (2·45 p.p.m.) in the presence of sodium and potassium in concentrations equivalent to the extreme ranges which may be found either in 1 : 10 serum or 1 : 50 muscle extracts.

These interference effects, being non-linear, are relatively enhanced the higher the dilutions used, which in the above instances approach the working limit of the instrument. In 1 : 10 serum the overall effect of sodium was approximately 12% of the magnesium reading. In undiluted urine and in faecal extracts, on the other hand, both because the concentrations are higher and because the proportion of sodium to magnesium tends to be lower, this type of interference is found to be insignificant.

The calibration curve of magnesium in these higher concentrations is shown in Fig. 1. This was obtained with a slit width of 0·08 mm. and a gain setting of 4·3. The shape of the curve is determined by a self-absorption phenomenon with increasing concentration.

In the estimation of magnesium traces, it is inevitable that the total background will be relatively high. The following figures obtained at very low sensitivity (slit 0·1 mm., gain 3·6) show that the magnesium in 1/10 serum contributes some 8% of the total light at 285·2 mμ.

Galvanometer reading with flame off	0
Galvanometer reading with deionized water	64
Galvanometer reading with 1/10 serum blank	64
Galvanometer reading with blank + Mg. 0·2 mEq. per litre	69·5

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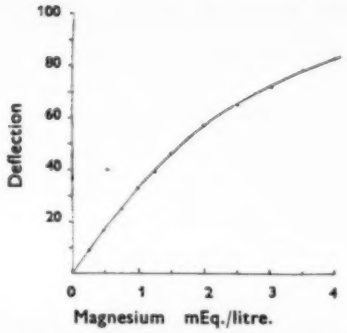

FIG. 1. *Magnesium standard curve, 0-4 mEq. per litre.*

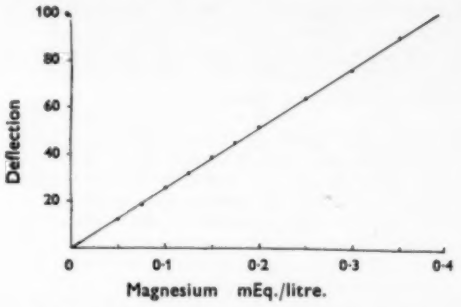

FIG. 2. *Magnesium standard curve, 0-0·4 mEq. per litre used for estimations in 1 : 10 serum.*

The accuracy and reproducibility of the estimation therefore depends to a large extent on the stability of background. The degree of this stability can be deduced from the figures in Table I.

TABLE I

RECOVERY OF ADDED MAGNESIUM IN SERUM

Sample	*Added Mg. (mEq. per litre)*	*Deflection*	*Recovery (mEq. per litre)*
Blank	—	0	—
Standard 0·1 mEq. per litre	—	26	—
"	0·150	62	0·140 (93%)
"	0·300	100	0·288 (99%)
Standard 0·16 mEq. per litre	—	41	—
"	0·150	78	0·144 (96%)
Blank	—	0	—
Serum A (1 : 10)	—	40	—
"	0·150	75	0·136 (91%)
Serum B (1 : 10)	—	46	—
"	0·150	83	0·144 (96%)
Serum C (1 : 10)	—	49	—
"	0·150	87	0·148 (99%)

TECHNIQUE

SERUM A stock serum blank solution is prepared by adding to 1 litre of deionized water sodium 140 mEq. (NaCl 8·19 g.), potassium 4 mEq. (K Cl 298 mg.), and calcium 5 mEq. ($CaCO_3$ 250 mg. dissolved in HCl). This solution is diluted 1 : 10 for use.

Standards are prepared by adding weighed amounts of magnesium sulphate to the undiluted blank to give a range from 0·5 to 4 mEq. per litre. This is diluted 1 : 10.

A minimum of 200 µl. serum is freshly diluted to 1 : 10 in deionized water and assayed at the magnesium atomic line of 285·2 mµ, using a slit width of 0·1 mm. and a gain setting of about 5·8 (Fig. 2).

Good recovery is obtained of added magnesium (Table I). Fig. 3 is a frequency histogram of normal sera assayed as described. This is at some variance with that of Wacker and Vallee (1957), but agrees with findings of

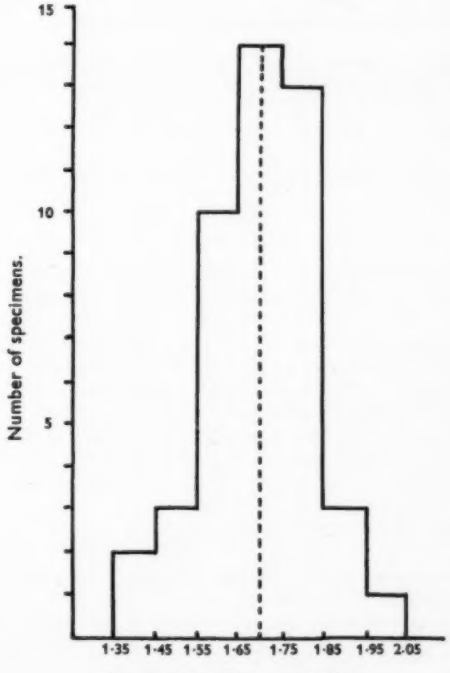

FIG. 3. *Frequency histogram of serum magnesium content of 46 normal sera from West Indian adults.*

MacIntyre and Davidsson (1958) and Van Fossan *et al.* (1959), using different instruments and techniques. The standard curve (Fig. 2) is more sensitive than that of Van Fossan *et al.*, who used an oxyhydrogen flame in a modified Beckman instrument.

MUSCLE AND RED CELLS Nitric acid digests of muscle in 1 : 50 dilution and red cell preparations lysed in a dilution of 1 : 25 may be estimated at approximately the same slit widths and gain settings as above. Magnesium estimations can thus be made in addition to other assays on muscle biopsy specimens of 50 to 150 mg. wet weight.

Nitric acid causes interference with the flame which becomes significant at concentrations around 0·1 N, and cannot be fully compensated by blank. The muscle should therefore be wet-ashed and partially homogenized in 0·2 N acid and diluted to a final concentration of 0·04 N.

The stock blank contains per litre K 15 mEq., Na 15 mEq., and PO_4 10 m.M., made by dissolving 1·065 g. K_3PO_4 and 1·725 g. $Na_3PO_4 \cdot 10H_2O$ in 1 litre, and is diluted 1 : 10 for use. Standards are prepared in the undiluted blank as for serum and are then diluted 1 : 10. The diluted blank represents a 1 : 50 dilution of the approximate electrolyte concentrations in the tissues (K 75 mEq., Na 75 mEq., and phosphate 50 m.M. per litre), and in practice permits accuracy over the full range of normal and abnormal tissue concentrations.

URINE Undiluted acidified urine may be assayed directly against deionized water. The standard curve in Fig. 1 is appropriate. In cases of magnesium deficiency, urinary magnesium may fall to very low levels, and may then be assayed against the standards for 1 : 10 serum using the serum blank.

FAECES Aliquots of faecal collections are evaporated overnight at 110° C., and are then treated with 6 N nitric acid in a boiling water bath. The digest is diluted, separated from fat, and centrifuged if necessary. The final acid concentration should not exceed 0·5 N and the appropriate amount of nitric acid must be added to standards and blank.

PRECAUTIONS

Care is required in maintaining the acetylene flow constant as it may be liable to minute but highly significant fluctuations. Another source of error in testing biological fluids is partial blockage of the atomiser. Both must be averted by frequent reference to the standards and blank. The modified atomiser enables clearance to be made by a stilette without interruption of the flame.

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The determination of magnesium in biological materials by flame photometry

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SYNOPSIS A method is described for the measurement of magnesium in biological materials and has been applied to the Unicam flame spectrophotometer SP 900.

Samples were diluted in 10 mM strontium ethylenedinitrolo tetraacetate (EDTASr), 10 mM potassium carbonate, giving final magnesium concentrations of about 0·1 to 0·2 mM. The EDTASr almost eliminated the depression of magnesium emission by interfering anions. The potassium carbonate slightly depressed both magnesium emission and flame background, which were then virtually unaffected by further addition of cations.

Spectral interference by sodium was controlled by including 14 mM sodium chloride in the plasma standards and making small corrections (usually under 2%) on the basis of the plasma sodium level. The urine standards contained no sodium, but a small correction (usually about 2%) was made on the basis of the urine sodium level. There was insufficient sodium in faeces to cause spectral interference.

Using the diluent described, added magnesium was satisfactorily recovered from plasma deproteinized with perchloric acid, urine, and faeces. When different volumes of identical samples of plasma and urine were analysed, the magnesium found was proportional to the volume of sample diluted.

The plasma magnesium levels reported for a small group of healthy people were similar to those found by previous workers.

In another paper (Fawcett and Wynn, 1961), we describe a method for the determination of calcium in biological materials by flame photometry, in which a releasing agent, magnesium sulphate, is used to release calcium emission from anion interference. We found that inorganic releasing agents were less effective when applied to magnesium emission. West and Cooke (1960), however, showed that ethylenedinitrolo tetraacetic acid (EDTA) reduced the interfering effects of anions on calcium emission, and was even more efficient in the case of magnesium. They added EDTA as the disodium salt, but sodium itself causes serious interference in magnesium estimations. We therefore tested the potentialities of other salts of EDTA.

Control of the effects of cations on the intensity of magnesium emission was sought by means which we had found satisfactory in the case of calcium emission, namely, the incorporation of a high concentration of a suitable cation.

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An additional problem in the measurement of magnesium is spectral interference by sodium, which Alcock, MacIntyre, and Radde (1960) solved, using their laboratory-made flame spectrophotometer. They reported that the Zeiss spectrophotometer PMQ II, with flame attachment, can be modified for use in the same way. This account concerns the determination of magnesium in biological materials with a less expensive, British-made flame spectrophotometer.

EXPERIMENTAL

APPARATUS The Unicam SP 900 flame spectrophotometer was used. It has a Meker-type burner utilizing acetylene and compressed air. The latter conveys the atomized solution from a separate spray chamber. The instrument is equipped with a fused prism monochromator and a photomultiplier detector.

The atomic emission line of magnesium, at 285·21 mμ, was used because of its greater selectivity than the molecular bands at 371 mμ and 383 mμ. The emission peak

was located by rotating the wavelength selector to give maximum deflection in the presence of magnesium. Sensitivity was found to be optimum using a lean fuel mixture (acetylene pressure, 10 cm. water; air pressure 28 lb./in²). With a richer mixture (acetylene pressure, 20 cm. water; air pressure, 28 lb./in²), sensitivity was decreased and the flame background was increased, but interference from anions was less. Initial experiments were carried out with both types of flame, but the leaner flame was chosen when it was found that anion interference could be controlled. The air pressure was not highly critical. The region in the flame of maximum emission was found with the centre of the slit 1 in. above the burner head.

The slit width was selected on the basis of the sensitivity required, and the effect of neighbouring emission lines. The only source of spectral interference near the magnesium line, at 285·21 mμ, is the almost coincident sodium line at 285·28 mμ. A slit width as low as 0·02 mm. corresponds to a nominal band width of 0·07 mμ, even assuming perfect optical characteristics, and therefore does not exclude participation by sodium. On the other hand, slight errors in reproducing the wavelength setting may cause random variations in the contribution to the total emission by sodium. A wider slit width ensures complete inclusion of both peaks, permitting predictable correction for sodium, and increases electronic stability. We found that avoiding exceptionally high electronic gain settings contributed more to instrumental stability than a high magnesium: flame background emission ratio. At a 0·08 mm. slit width, this ratio was 0·2 with 0·2 mM magnesium, the nominal band width was 0·3 mμ, and sensitivity was adequate for 1 : 10 plasma dilutions.

GALVANOMETER READINGS The calibration curves in Fig. 1 show that emission was more nearly proportional to concentration in the range 0 to 0·2 mM than in the range 0 to 0·5 mM. There were significant day-to-day variations in reproducing the second curve but not the first.

Blank and standard readings were checked between every two or three readings of unknowns. Each solution was read at least four times, and the mean of the middle two readings was calculated. If these two differed by more than 1%, further readings were obtained.

When special diluting solutions were used, standard magnesium solutions were diluted in the same diluent as the samples, and this diluent was also used for setting zero on the galvanometer.

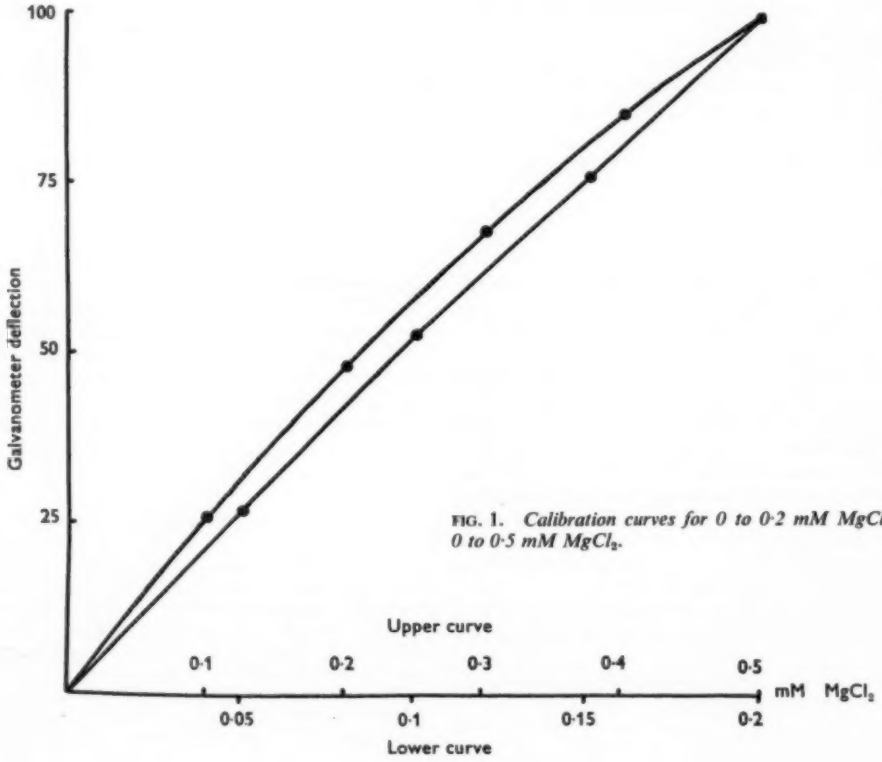

FIG. 1. *Calibration curves for 0 to 0·2 mM $MgCl_2$ and 0 to 0·5 mM $MgCl_2$.*

PREPARATION OF SOLUTIONS Solutions were prepared from spectroscopically pure reagents, or high purity alternatives, and deionized water.

Stock solutions containing 0·1 M magnesium were prepared in two ways: (1) from weighed magnesium ribbon dissolved in the minimum of 1·0 N hydrochloric acid; (2) from weighed magnesium sulphate dried to constant weight at 300°C. When sulphate interference was controlled, as described below, 1 : 500 dilutions of these two solutions gave identical readings.

PREPARATION OF SAMPLES Samples were diluted in duplicate to give a final magnesium concentration of about 0·1 to 0·2 mM.

Plasma samples were obtained from heparinized blood. Deproteinization was necessary because the viscosity of 1 : 10 plasma dilutions reduced the flow rate through the atomiser by about 6%, and this varied from one sample to another. An acid protein precipitant was used to ensure separation of protein-bound magnesium, and its concentration was kept as low as possible to minimize interference effects. Two millilitres of plasma were deproteinized with 2 ml. of 0·5 M perchloric acid and allowed to stand for 10 minutes. Two millilitres of plasma contain only about 1·85 ml. of water, and therefore the solid content contributes an average error of 0·15 ml. in 4 ml., or 4%. This error was reduced to 0·15 ml. in 16 ml., or 1%, by adding 12 ml. of water before centrifuging for 10 minutes at 3,000 to 3,500 r.p.m. The supernatant solution corresponded to a 1 : 8 plasma dilution, with a 1% correction for solids.

Twenty-four-hour urine samples were preserved with 10 ml. of hydrochloric acid, and diluted 1 : 25.

Faeces were prepared by homogenizing with water, weighing, and transferring weighed portions of about 2g. to nickel crucibles of 50 mm. diameter. The contents were dried at 105° and ashed overnight in the uncovered crucibles at 420 to 450°. It was found that magnesium was lost at higher temperatures. The ash was dissolved by boiling carefully with 1 ml. of 1·0 N hydrochloric acid, transferred quantitatively and diluted to 100 ml. This solution was further diluted 1 : 5.

SPECTRAL INTERFERENCE FROM SODIUM Under the conditions described, 14 mM sodium chloride gave the same emission as 0·015 mM magnesium chloride, and this interference was directly proportional to sodium concentration. Thus in plasma the emission due to sodium represented 15 to 20% of the emission due to magnesium. In urine, which nearly always has a much lower sodium : magnesium ratio, the sodium emission was usually about 2 to 3% of the magnesium emission. The sodium content of faeces caused negligible interference.

There are three methods of controlling spectral interference by sodium: (1) chemical separation of magnesium from sodium; (2) inclusion of the same concentration of sodium chloride in the magnesium standards as its concentration in the diluted sample; (3) correction of observed magnesium readings on the basis of the sodium concentration, and of its known interference.

The first of these alternatives is time-consuming and introduces additional sources of error. The second was adopted for plasma because of its relatively constant sodium content. A concentration of 14 mM sodium chloride was incorporated in the plasma standards, and if the plasma sodium level was known to differ significantly from 140 mM, the observed magnesium level was adjusted by 0·01 mM (about 1%) for each 10 mM difference.

The third method of correction was adopted for urine because sodium interference was much smaller but more variable. For each 10 mM sodium the urine contained, 0·01 mM was subtracted from the observed magnesium result.

EFFECTS OF ADDED SUBSTANCES ON FLAME BACKGROUND Each of the following solutions was found to have no measurable effect on flame background at 285·2 mμ, under the conditions described: 10 mM hydrochloric acid, 10 mM sulphuric acid, 10 mM phosphoric acid, 10 mM perchloric acid, 10 mM ammonia, 1 mM calcium chloride, and 5 mM glucose.

On the other hand, we found flame background was suppressed by potassium salts, 20 mM potassium chloride cancelling the emission from 0·012 mM magnesium chloride. Further increments of potassium concentration had less effect in suppressing flame background, so that 40 mM potassium chloride cancelled the emission from only 0·017 mM magnesium chloride.

The potassium concentration in plasma and faeces was insufficient to affect the flame background, but in urine was sufficient to cause errors of 1 to 2%. When the diluting solution contained potassium, the effect of potassium in the sample was less.

Urea in pure solution also suppressed flame background but this suppression was reduced in the presence of other solutes and was negligible in urine dilutions.

EFFECTS OF ADDED SUBSTANCES ON MAGNESIUM EMISSION Preparing the samples as described, the anions causing depression of magnesium emission were phosphate, sulphate, and perchlorate. It was found that, in contrast to the enhancement of calcium emission by other cations, magnesium emission was depressed by sodium, potassium, and calcium. The effects of these ions were cumulative, so that recoveries of magnesium added to urine were only about 80%.

The effect of EDTA salts, and of strontium chloride, on magnesium emission was tested before investigating their efficiency in controlling anion interference. The disodium, dipotassium, diammonium, and ferric complexes of EDTA, and strontium chloride, each depressed magnesium emission. We found, however, that the strontium salt of EDTA (EDTASr) had no effect on magnesium emission, and almost eliminated depression by added anions. Also we found that EDTASr had no effect on flame background. A concentration of 5 mM was adequate to control expected concentrations of interfering anions, but a concentration of 10 mM was adopted to permit a margin of safety.

Although cation interference with magnesium emission was different in direction from its interference with calcium emission, and therefore presumably different in mechanism, it seemed possible that it might reach a limiting level in the presence of excess of an interfering

cation, as it does with calcium (Fawcett and Wynn, 1961). As we had already noted one advantage of incorporating potassium in the diluting solution, because it reduced the effect on flame background of potassium in the sample, and as its depression of magnesium emission was slight, we investigated the effects of potassium added in the form of chloride, perchlorate, hydroxide, and carbonate. These were found equally efficient in maintaining a plateau level of potassium interference and in almost eliminating depression by sodium and calcium.

The choice of which potassium compound to incorporate in the diluting solution was based upon the necessity for simultaneous control of *p*H. It was found that magnesium was precipitated at high *p*H levels and that free EDTA was precipitated at low *p*H levels. A concentration of 10 mM potassium carbonate not only provided the potassium level required to control cation interference but also maintained the *p*H of urine and faecal dilutions between 6 and 11. Additional potassium carbonate was necessary to neutralize the perchloric acid in deproteinized plasma.

CONTROL OF INTERFERENCE EFFECTS Table I shows the effects of interfering substances on magnesium emission when these were added to 0·2 mM magnesium chloride at concentrations greater than those likely to be encountered using the dilutions described. In the presence of 10 mM EDTASr, 10 mM K_2CO_3, these effects were reduced to very low levels. The cumulative depression of magnesium emission by a mixture of interfering substances, and its control in the presence of the diluting solution, are also illustrated in Table I.

TABLE I

DEPRESSION OF EMISSION FROM 0·2 mM $MgCl_2$ BY INTERFERING SUBSTANCES

Interfering Substance	*Concentration (mM)*	*Percentage Depression*	
		Without Control	*With Control*[1]
NaCl	3	3	1
	15	5	1
KCl	10	2	0
	20	3	0
	30	3	0
$CaCl_2$	0·5	2	0
	1	3	1
H_3PO_4	2	6	0
	10	6	1
H_2SO_4	2	4	0
	10	4	1
$HClO_4$	50	11	1
	100	11	1
HCl	10	1	1
NaCl	15		
KH_2PO_4	10		
K_2SO_4	10	20	2
$CaCl_2$	1		

[1]Interference was controlled by incorporating 10 mM EDTASr, 10 mM K_2CO_3, in the diluting solution.

The diluting solution reduced the effect of any further addition of potassium on flame background, so that with a urine sample containing 50 mM potassium, the necessary correction of the observed urine magnesium level was equivalent to only 0·01 mM (usually less than 1%).

DILUTING SOLUTIONS Two stock diluting solutions were prepared: A, 0·05 M EDTASr, 0·25 M K_2CO_3, and B, 0·25 M EDTASr, 0·25 M K_2CO_3. Solution A was used for plasma, solution B for urine and faeces.

WORKING STANDARDS Two series of standards were prepared, one for plasma analysis and one for use with urine and faeces. In each series, magnesium concentrations of 0·05 mM, 0·10 mM, 0·15 mM, and 0·20 mM were obtained by dilution of a stock standard solution. The plasma standards incorporated 14 mM sodium chloride, 50 mM perchloric acid, and a 1 : 5 dilution of diluting solution A. The other series of standards incorporated a 1 : 25 dilution of diluting solution B.

PLASMA Ten millilitres of the prepared supernatant, corresponding to a 1 : 8 plasma dilution, were mixed with 2·5 ml. of diluting solution A. The final EDTASr concentration was therefore 10 mM, while the concentration of potassium carbonate was sufficient to neutralize the perchloric acid precipitant. Originally it was intended to precipitate the potassium perchlorate by cooling, but this extra step was omitted when it was found that depression of magnesium emission by potassium perchlorate was almost eliminated in the presence of the diluting solution.

The total plasma dilution was 1 : 10. Deproteinization introduces error due to the solid content of plasma but it has been calculated above that, under these conditions, this increased the readings by 1% or less. With the diluting solution and standards described, it appeared from the results in Table I that interfering substances in plasma depressed magnesium emission by 1% or less. These two small errors, operating in opposite directions, were neglected.

URINE AND FAECES One millilitre of urine, or 5 ml. of faecal ash solution, was mixed with 1 ml. of diluting solution B, and diluted to 25 ml. The final EDTASr and potassium carbonate concentrations were each 10 mM.

RESULTS

In the absence of a generally accepted method of measuring magnesium in biological materials we depended upon internal methods of assessing the reliability of the present method, namely, recovery experiments and serial dilutions of identical samples.

The results of 12 recovery experiments on plasma are reported in Table II. Magnesium sulphate was added to each plasma sample before deproteinization, raising the concentration by 1·0 mM. The mean recovery was 99·1% and the scatter was small. Even better results were obtained in the recovery experiments reported in Table III, in which the magnesium

TABLE II

RECOVERY OF 1·0 mM MAGNESIUM ADDED TO EACH OF 12 PLASMA SAMPLES

Plasma Mg (mM)	*Plasma + Added Mg (mM)*	*Percentage Recovery*
0·62	1·61	99
0·63	1·60	97
0·66	1·64	98
0·82	1·80	98
0·83	1·82	99
0·83	1·84	101
0·84	1·81	97
0·86	1·84	98
0·91	1·93	102
0·91	1·94	103
0·95	1·93	98
0·95	1·94	99
		Mean 99·1
		S.D. 1·9

TABLE III

RECOVERY OF 2·0 mM MAGNESIUM ADDED TO EACH OF 10 URINE SAMPLES

Urine Mg (mM)	*Urine + Added Mg (mM)*	*Percentage Recovery*
0·84	2·83	99·5
1·59	3·58	99·5
1·60	3·63	101·5
1·78	3·80	101
1·90	3·90	100
2·20	4·18	99
2·28	4·26	99
2·90	4·86	98
3·03	5·05	101
3·35	5·33	99
		Mean 99·7
		S.D. 1·1

levels of 10 urine samples were raised by 2 mM, and in Table IV, in which magnesium was added to samples of faeces. In the recovery experiments on faeces, magnesium was added sometimes before ashing and sometimes afterwards, the results being reported separately.

A further check was made by analysing several different volumes of identical plasma and urine samples. Fig. 3 shows that when 1 ml., 2 ml., 3 ml., and 4 ml. portions of the same plasma sample were treated as described above, adjusting the volume of water added after deproteinization to keep the final dilution factor constant, the magnesium results

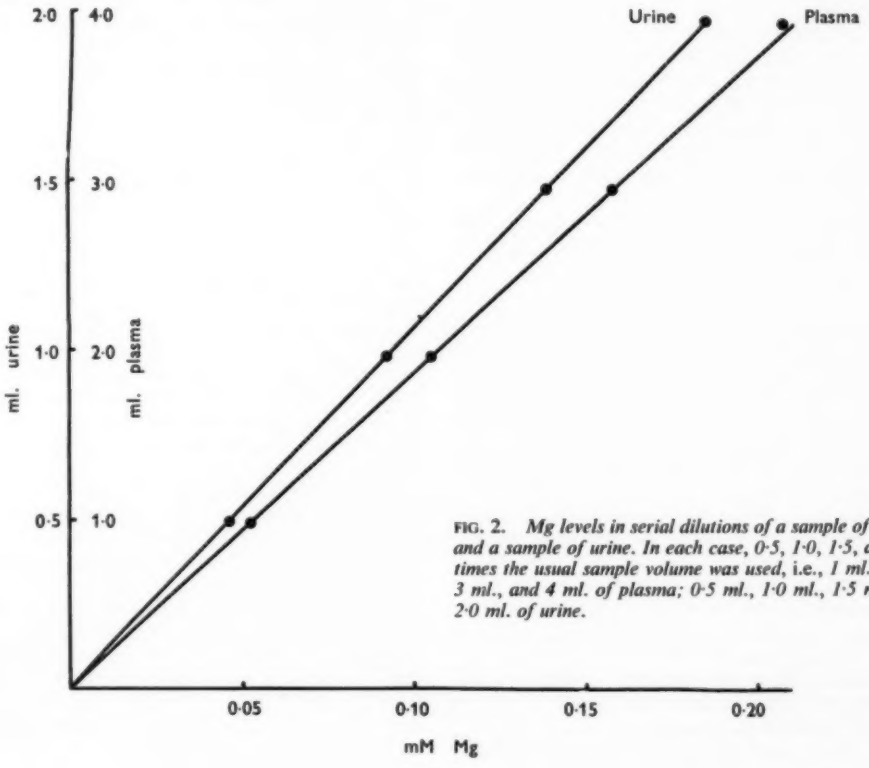

FIG. 2. *Mg levels in serial dilutions of a sample of plasma and a sample of urine. In each case, 0·5, 1·0, 1·5, and 2·0-times the usual sample volume was used,* i.e., *1 ml., 2 ml., 3 ml., and 4 ml. of plasma; 0·5 ml., 1·0 ml., 1·5 ml., and 2·0 ml. of urine.*

TABLE IV

RECOVERY OF MAGNESIUM ADDED TO SAMPLES OF FAECES TO RAISE Mg LEVEL IN ASH SOLUTION BY 0·5 mM

Initial Faecal Mg[1] *(mM)*	*Percentage Recovery*	
	(a)	*(b)*
0·22	—	101
0·26	98	101
0·27	100	—
0·32	102	—
0·35	101	99
0·37	100	—
0·40	101	98
0·40	—	101
0·41	—	99
0·41	102	—
0·42	99	99
0·42	100	—
0·42	—	99
0·47	100	98
0·52	98	—
0·52	—	99
0·54	99	101
0·58	—	99
Mean	100·0	99·5
S.D.	1·3	1·2

[1]In terms of concentration in faecal ash solution

(*a*) Mg added before ashing. (*b*) Mg added after ashing.

obtained were in proportion to the volume of plasma used. Fig. 2 also shows that when 0·5 ml., 1·0 ml., 1·5 ml., and 2·0 ml. portions of the same urine sample were diluted to 25 ml., the magnesium results obtained were proportional to the volume of urine analysed. These internal checks on the methods used confirmed that interference effects were adequately controlled.

The mean plasma magnesium level found in 10 healthy subjects was 0·87 mM (S.D. 0·035 mM).

DISCUSSION

In the measurement of magnesium, flame photometry has distinct advantages over absorptiometric procedures, which are of limited accuracy, or titration with EDTA, which involves either prior separation of magnesium from calcium or calculation from the difference of two titration figures. On the other hand, the newer technique of flame atomic absorption spectroscopy has several advantages over flame emission. It is more sensitive and spectral interference by sodium is avoided. But indirect interference by anions and cations, affecting the absorption by magnesium itself, must be controlled by methods similar to those described for flame emission. As there is no overall advantage in replacing flame emission by flame absorption for the measurement of sodium, potassium, and calcium, economic reasons may dictate the choice of flame emission for magnesium also.

The special problems in measuring magnesium by flame photometry are its much lower emission energy, even than calcium, the spectral interference by sodium, the effects of solutes on flame background, and indirect interference effects altering the emission by magnesium itself.

Van Fossan, Baird, and Tekell (1959) increased magnesium emission by incorporating high concentrations of organic solvents, but in our experience this decreased flame stability, presented explosion risks because of the separate spray chamber, and necessitated special precautions to prevent the evaporation of solutions.

The molecular bands of magnesium have greater emission intensity than the atomic resonance line at 285·2 mμ and were used in most of the early work on the determination of magnesium in biological materials. Instrumental improvements in sensitivity permit measurement at the much more selective atomic line, as has been shown by Alcock *et al.* (1960).

The instrument and conditions employed by us differed from those of Alcock and her colleagues in several respects. The instrument was designed to use compressed air instead of oxygen. While this was more convenient, there was some loss of sensitivity. This in turn, together with the difficulty of ensuring exactly reproducible wavelength settings with the extremely narrow slit width of 0·02 mm. selected by Alcock *et al.* (1960), determined our choice of a 0·08 mm. slit width. Our magnesium: flame background emission ratio was consequently lower, and fluctuation of flame background could contribute substantially to instrumental instability. We found, however, that total stability was satisfactory, and as good using 0·2 mM as using 0·5 mM magnesium, with its correspondingly higher magnesium: flame background emission ratio. This ratio, considered in isolation, is thus not an indication of instrumental performance.

Spectral interference by sodium was greater with a 0·08 mm. slit width than with one of 0·02 mm., but it was easy to control when the sodium concentration was known. Using the dilution procedure described, even if the plasma sodium level was not known, variation within the range 120 to 160 mM contributed errors of less than 2%. If correction for urine sodium was not made, errors in magnesium results were usually about 2%, and were less if an average figure of, say, 50 mM sodium was assumed.

Using the dilution methods described, potassium was the only biological solute found to affect flame background and this effect was not measurable with the concentration of potassium in plasma or faeces. In urine, correction on the basis of the known potassium content rarely exceeded 1% of the magnesium level.

Indirect interference effects were almost entirely eliminated by dilution in a solution containing the strontium salt of EDTA and potassium carbonate. This was preferred to attempting to create the same degree of interference in the standards as in the unknowns by synthesizing complex standard solutions with a composition approximating to that of the solutions analysed (Alcock *et al.*, 1960).

The strontium salt of EDTA was more efficient in maintaining magnesium emission than other EDTA salts, and unlike the disodium salt, caused no spectral or background interference. Its efficiency was also greater than that of strontium chloride, which was among the inorganic releasing agents we had tested previously, and this demonstrated that its action was not due merely to the releasing agency of strontium.

The method described here has been found satisfactory in the analysis of other materials, such as diets, bone, and soft tissues. As none of these presents greater interference problems than plasma and urine, results of recovery experiments have not been included.

The plasma magnesium levels found in healthy subjects, although too few to establish a normal range, are similar to the levels reported by Van Fossan *et al.* (1959) and Alcock *et al* (1960).

We would like to express our gratitude to Professor N. F. Maclagan and to the British Empire Cancer Campaign for making available to us the flame spectrophotometer used in this work.

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Plasma magnesium in health and disease

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SYNOPSIS Simpler and more accurate flame spectrophotometric techniques are now available for estimating magnesium. Because of the development of modern electrolyte therapy, cases of hypomagnesaemia are now encountered more frequently. The condition may lead to dangerous convulsions without sufficient warning. Some causes of hypomagnesaemia and hypermagnesaemia are presented.

Studies of plasma magnesium levels have not yet been widely introduced to the field of routine clinical chemistry. However, two factors have aroused interest in studying the plasma level of this element in recent years. 1, Just as the adoption of therapeutic measures for treating dehydration and disturbances of sodium metabolism in the past revealed cases of hypokalaemia and emphasized the importance of studying plasma potassium, so the treatment of disturbances of potassium, sodium, and calcium metabolism is now unmasking cases of hypomagnesaemia. 2, The old methods of estimating magnesium, which were either cumbersome or inaccurate, are now superseded by simple flame-photometric techniques.

Magnesium deficiency has been thoroughly studied in rats. Briefly this condition results in peripheral vasodilatation, convulsions, a rise in plasma calcium, and nephrocalcinosis (MacIntyre and Davidsson, 1958).

The condition has also been reported in man. Because it is usually associated with multiple electrolyte and nutritional deficiencies the syndrome is not yet clearly defined. However, convulsions, which may be fatal, have been reported (Hanna, Harrison, MacIntyre, and Fraser, 1960).

Using simple and accurate flame spectro-photometric techniques, magnesium metabolism was studied in about 100 subjects at the Hammersmith Hospital, London, over the last three years. In this paper an attempt is made to summarize and discuss the plasma magnesium findings in some of them.

MATERIAL AND METHODS

Subjects were studied in the metabolic unit. The balance technique and analytical methods have been reported elsewhere (Hanna *et al.*, 1960). Plasma magnesium was determined by the method described by Alcock, MacIntyre, and Radde (1960) and involved diluting 10 times with a deproteinizing fluid and interpolating flame-spectrophotometric emission of the supernatent between suitable standards. The flame-spectrophotometer used was a Zeiss P.M.Q. II spectrophotometer[2] with a flame attachment and a double monochromator.

RESULTS AND DISCUSSION

NORMAL LEVEL AND RANGE Fig. 1 shows the mean plasma magnesium levels of 12 normal healthy laboratory workers. A histogram showing plasma magnesium levels in 72 normal subjects as reported by Alcock *et al.* (1960) is also charted. It can be seen that the mean plasma magnesium level for normal subjects is 1·66 mEq./litre and that the range of normal is about 1·5 to 1·8 mEq./litre.

Table I summarizes the mean normal plasma magnesium level as reported by different authors. The differences between the mean values reported do not reflect a wide range for normal plasma magnesium. Studies in magnesium metabolism show that in health the body maintains the plasma magnesium level just as carefully as it does calcium (MacIntyre and Wootton, 1960). FitzGerald and Fourman (1956) put themselves on a magnesium-deficient diet (1·1 mEq. per day) for about four weeks and notwithstanding found no change in their plasma magnesium levels. Similar findings were reported by Walker and Walker (1936).

The differences between the means reported seem to be due to the fact that some authors used magnesium oxide as a standard. This substance is not suitable because it is difficult to prepare in a pure state and it absorbs carbon dioxide from the air

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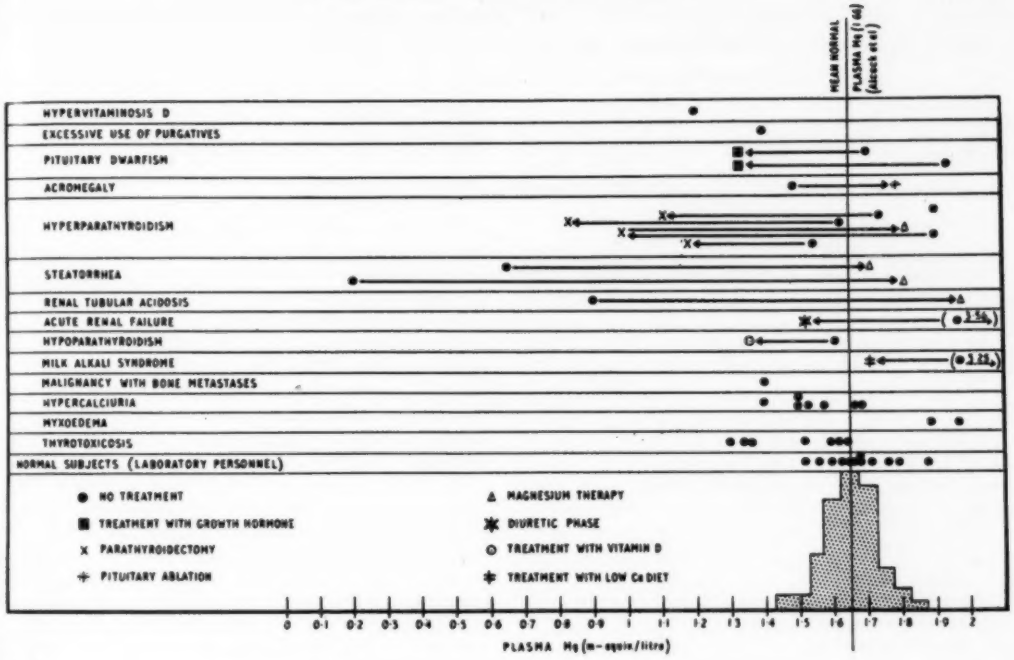

FIG. 1

TABLE I

MEAN PLASMA MAGNESIUM LEVELS REPORTED BY VARIOUS AUTHORS

Author	*Method*	*Mean (mEq./l.)*
Watchorn and McCance (1932)	P.P.	2·07
Hald (1933)	P.P.	1·70
Greenberg, Lucia, Mackey, and Tufts (1933)	H.P.	2·28
Schachter (1959)	F.	1·83
Hoffman (1937)	H.P.	1·82
Hirschfelder and Haury (1938)	T.Y.	1·76
Cope and Wolff (1942)	P.P.	1·66
Simonsen, Westover, and Wertman (1947)	P.P.	1·68
Silverman and Gardner (1954)	T.Y.	1·77
Davis (1955)	F.P.	1·66
Carr and Frank (1956)	E.D.T.A.	1·72
Wacker and Vallee (1957)	F.P.	2·05
Hunter (1958)	E.B.	1·69
Van Fossan, Baird, and Tekell (1959)	F.P.	1·67
Alcock *et al.* (1960)	P.F.	1·66

P.P. = phosphate precipitation methods
T.Y. = titan yellow methods
H.P. = hydroxyquinoline precipitation methods
F.P. = flame photometric methods
E.D.T.A. = titration with E.D.T.A.
E.B. = erichrome black methods
F = fluorimetric methods

heating to 900°C. is needed to decompose magnesium carbonate (Mellor, 1923). Using impure magnesium oxide heated to 110° or to 120°C., as some authors did (Wacker and Vallee, 1957), would produce high results.

HYPOMAGNESAEMIA Table II summarizes some of the wide variety of clinical conditions reported to be associated with hypomagnesaemia. However, before accepting some of these reports in terms of a causal relationship, certain points should be taken into consideration.

If liver cirrhosis is accepted as one of the causes of hypomagnesaemia (Flink, Konig, and Brown, 1955), it, in association with alcoholism (Flink, Stutzman, Anderson, Konig, and Fraser, 1954), may well be the result of the liver cirrhosis frequently associated with alcoholism. Also the reported association between idiopathic epilepsy and hypomagnesaemia (Haury and Cantarow, 1942) should be reinvestigated to see whether the subjects are genuinely idiopathic or are just hypomagnesaemic patients showing convulsions. Such considerations, however, do not alter the fact that hypomagnesaemia may result from certain clinical conditions, especially

TABLE II

CLINICAL CONDITIONS REPORTED TO BE ASSOCIATED WITH HYPOMAGNESAEMIA

Clinical condition	*Authors*
Hyperparathyroidism	
Pre-operative	Harmon (1956); Barnes, Crane, and Cope (1957)
Post-operative	Potts and Roberts (1958); Agna and Goldsmith (1958)
Primary aldosteronism	Mader and Iseri (1954); Milne, Muehrcke, and Aird (1957)
Gastrointestinal disturbances (malabsorption syndrome, vomiting, resection, etc.)	Hammarsten and Smith (1957); Card and Marks (1958); Randall *et al.* (1959); Vallee *et al.* (1960); Hanna *et al.* (1960); MacIntyre, Hanna, Booth, and Read (1961)
Osteolytic bone disease	Smith and Eliel (1956)
Idiopathic hypercalcaemia in infancy	Lowe, Henderson, Park and McGreal (1954)
Chronic glomerulonephritis	Martin *et al.* (1952); Randall *et al.* (1959)
Alcoholism	Flink, *et al.* (1954); Randall *et al.* (1959)
Liver cirrhosis	Flink *et al.* (1954)
Eclampsia, epilepsy, diabetes mellitus, bronchial asthma, malignancy, thyrotoxicosis	Haury (1940); Haury and Cantarow (1942)

gastrointestinal disturbances, and may be in itself hazardous to the patient's health.

Fig. 1 shows the means of plasma magnesium levels for 45 subjects studied; 12 of these were normal laboratory workers and the rest were selected because they were expected to show some disturbance in magnesium metabolism. Accepting the range of 1·5 to 1·8 mEq./l as the normal range of plasma magnesium, 16 of these subjects were hypomagnesaemic or became hypomagnesaemic after certain therapeutic measures were taken. These cases of hypomagnesaemia (Fig. 1) are attributed to the following clinical conditions:

Clinical Condition	*No. of Cases*
Removal of parathyroid adenoma	4
Thyrotoxicosis	3
Steatorrhoea	2
Administration of vitamin D	2
Treatment with growth hormone	2
Renal tubular acidosis	1
Acromegaly	1
Excessive use of purgatives	1
Malignancy with bone metastases	1
Hypercalciuria (idiopathic)	1

These cases may be divided into two groups according to their aetiology. The first includes clinical conditions in which magnesium is lost from the extracellular fluid compartment to bone and soft tissues, and also removal of parathyroid adenoma and administration of vitamin D or growth hormone (Hanna, 1960, 1961; Hanna, Harrison, MacIntyre, and Fraser, 1961). Patients in this group may show a positive magnesium balance. The second group includes clinical conditions in which magnesium is lost from the body via the gastrointestinal tract and the kidneys. Here a negative magnesium balance is the cause of hypomagnesaemia. Cases of primary aedosteronism, steatorrhoea, idiopathic hypercalciuria, thyrotoxicosis, renal tubular acidosis, malignancy with bone metastases, and excessive use of purgatives fall into this group (Hanna *et al.*, 1960; Hanna and MacIntyre, 1960).

Clinical picture An understanding of the clinical picture of hypomagnesaemia is of importance to the clinician in order to be able to identify cases of hypomagnesaemia. Previously, such cases were not diagnosed, mainly because of the lack of suitable analytical techniques but partly because patients died, suffering from shock or dehydration, long before the development of hypomagnesaemia. But now, with the development of suitable analytical methods for magnesium, together with the advancements in the field of fluid and electrolyte replacement therapy and the development of modern surgical and medical life-saving techniques, cases of hypomagnesaemia are being discovered more frequently.

The syndrome of magnesium deficiency has been well studied in experimental animals (MacIntyre and Davidsson, 1958). In farm ruminants magnesium deficiency presents an economic problem, and its incidence in suckling calves has been placed as high as 5% in Great Britain (Blaxter and Sharman, 1955). The syndrome is characterized by convulsions. When it occurs in lactating cows it is sometimes called 'grass staggers'.

However, in man the syndrome is less clearly defined. Tetany, tremors, choreiform movements, fibrillary twitches, delirium, rigidity, confusion, status epilepticus, cerebellar symptoms, stupor, and athetoid movements are amongst the signs and symptoms reported in association with hypomagnesaemia in man. Of these, the most widely accepted is tetany and several textbooks describe 'hypomagnesaemic tetany' as a clinical entity (Duncan, 1952; Nelson, 1959). Despite this wide variety of signs and symptoms, Martin, Mehl, and Wertman (1952) failed to elicit any due to hypomagnesaemia in a study of a series of 16 patients with plasma magnesium levels less than 1 mEq./l. and they particularly emphasized the absence of tetany.

Confusion regarding tetany has arisen from two sources. Although the widely accepted definition is the development of peripheral painful carpopedal spasms, some authors have not distinguished between

TABLE III

SIGNS AND SYMPTOMS SOMETIMES ENCOUNTERED IN HYPOMAGNESAEMIA

Clinical Condition	*Hospital No.*	*Plasma Mg. (mEq./l.)*	*Signs and Symptoms*
Steatorrhoea	227935	0·2	Vertigo, muscle weakness, positive Chvostek's sign, low alkaline phosphatase, low voltage of E.E.G., ataxia, convulsions
Hyperparathyroidism (post-operative)	220252	1·0	Muscle weakness, mental disturbances
Renal tubular acidosis	199012	0·9	Positive Chvostek's sign, convulsions

convulsions and tetany (Vallee, Wacker, and Ulmer, 1960). The second source of confusion is that Kruse, Orent, and McCollum (1932), followed by Greenberg and Tufts (1938), used the term 'tetany' to describe the convulsions occurring in magnesium-deficient rats. The latter authors used the term, despite their recognition of the clear distinction between the neuromuscular disturbances produced by hypocalcaemia and those produced by hypomagnesaemia. They stated that 'magnesium tetany is of a definitely different type from low calcium tetany'. This use of one term for two distinct conditions has led to a search for hypomagnesaemia in patients suffering from all types of neuromuscular disturbances. It is hardly surprising, therefore, that low plasma magnesium was sometimes encountered, since most of these patients were suffering from multiple deficiencies (Randall, Rossmeisl, and Bleifer, 1959).

Even the control of tetany or any other neuromuscular disturbance by magnesium does not establish a causal relationship between magnesium deficiency and tetany. Harrison, Fraser, and MacIntyre (unpublished data) found that an intravenous infusion of magnesium may control tetany produced by hypocalcaemia in the presence of normal plasma magnesium levels.

The danger of accepting the reported signs and symptoms is the false sense of security in their absence. Out of the 16 cases of hypomagnesaemia presented in Fig. 1 only three showed signs and symptoms attributable to hypomagnesaemia (Table III). In two of them the earliest striking manifestation of hypomagnesaemia was an attack of epileptiform convulsions.

All the signs and symptoms listed in Table III were reversed by magnesium infusion. Except for the convulsive seizures, the other signs and symptoms were not striking.

The convulsive seizures In Case A (Table III), 12 days after admission the patient suddenly vomited and became confused and disorientated. Two convulsive epileptiform seizures followed. An intravenous infusion of 100 mEq. magnesium chloride in 1 litre 5% dextrose was given over the next four hours. During the day the confusion receded and a left homonymous hemianopia became evident along with some motor dysphasia, perseveration, and right-sided weakness and inattention. An E.E.G. showed changes suggesting a focal cerebral lesion. All these neurological signs gradually disappeared over the next five days. Subsequently, the patient had complete amnesia for the convulsive seizures and the day following them.

Case C (Table III), six days after admission, had a generalized epileptiform convulsion. An E.E.G. showed low voltage for all complexes. Chvostek's sign was positive. An intravenous infusion of 50 mEq. magnesium chloride was given over four hours. At the end of the infusion the patient remarked that she felt better and stronger. Chvostek's sign became negative and an E.E.G. taken 45 minutes after the end of magnesium infusion showed a definite increase in voltage of the Q.R.S. complex and T waves.

It is not possible, at this stage, to claim which of the signs and symptoms is due to hypomagnesaemia and which to magnesium deficiency in other compartments of the body. Muscle weakness and a low voltage in the E.C.G. could have resulted from intracellular magnesium deficiency. Radde, Hanna, and MacIntyre (unpublished data) found that magnesium-deficient rats show a low voltage in the E.C.G. when compared with normal controls.

These cases illustrate the mildness of the signs and symptoms in magnesium deficiency and show that very little warning is given before dangerous convulsions ensue.

HYPERMAGNESAEMIA Hypermagnesaemia was reported in cases of uraemia. Hamburger (1957) reported that clouding of consciousness associated with uraemia follows fairly closely the level of plasma magnesium.

In the present study (Fig. 1) a mild degree of hypermagnesaemia was encountered in two cases of myxoedema, in two cases of hyperparathyroidism with renal damage, and in one case of pituitary dwarfism. A severe degree of hypermagnesaemia was encountered in one case of the milk-alkali syndrome and in one of acute renal failure. Both patients returned to normal, the first after being put on a low-calcium diet, the second in the diuretic phase.

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A possible source of error in the diagnosis of phaeochromocytoma

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SYNOPSIS The finding is reported of an apparent high level of urinary catechol amines in a child with pyelonephritis. As a result of investigations the source of error was traced to tetracycline therapy.

The biochemical estimation of catechol amines in the urine is now an established method of diagnosing phaeochromocytoma (Engel and von Euler, 1950; Goldenberg, Serlin, Edwards, and Rapport, 1954). Though more accurate than tests involving the use of adrenolytic drugs such as dibenyline, piperoxane (benzodioxane), and phentolamine (rogitine) false positive results have occasionally been reported. The recent description (Malbin, 1959) of a patient, whose urine contained 10 times the normal concentration of catechol amines but in whom no adrenal tumour could be found, prompts the description of the following case history and observations.

CASE REPORT

E.P., a girl aged 3 years 7 months, attended the Royal Belfast Hospital for Sick Children in April 1957, because of recurrent attacks of otitis media. There was no history of any other illness. Her blood pressure was 95/60 mm. Hg. Albuminuria was detected on routine testing. The urinary deposit showed a few pus cells only; culture was sterile on three occasions. The blood urea was 21 mg. per 100 ml.

Towards the end of May she developed polydypsia, cardiac enlargement (confirmed radiographically), and hypertension (blood pressure 180/130 mm. Hg). The fundi were normal in appearance. Femoral pulsation was felt.

The urine contained protein, 0·1 to 0·2 g. %, and pus cells 5 to 15 per high power field (centrifuged deposit). Two specimens were sterile; one specimen on culture yielded growth of *B. coli* sensitive to oxytetracycline.

Blood urea was 24 mg. per 100 ml.

Intravenous pyelography showed the soft tissue outline of the right kidney to be smaller than the left. Dye was well secreted on both sides. Neither the pelvis nor the calyces were distorted.

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The patient was thought to have chronic pyelonephritis and on 6 June 1957 was started on a course of oxytetracycline 125 mg. six-hourly. The catechol amine content of urine collected on 20 June was reported as 470 micrograms per 24 hours (Hingerty's method, 1957; normal value = 40 μg./24 hours). This high level suggested a phaeochromocytoma as the cause of the hypertension. However, an intravenous phentolamine (rogitine) test produced no fall in blood pressure. This observation cast doubt on the validity of the urine catechol amine result and this was accordingly re-estimated. On this second occasion a value of 67 micrograms per 24 hours was obtained. (Antibiotic therapy had been discontinued one week previously.)

In early July the patient's condition deteriorated rapidly and cardiac failure developed. Improvement followed treatment with digoxin, mecamylamine, and a low-salt diet though hypertension persisted (blood pressure 170/120 to 200/140 mm. Hg).

On 31 July 1957 a right-sided nephrectomy was performed. The kidney, which was grossly scarred and contracted, showed on histological examination active and extensive chronic pyelonephritis and marked endarteritic vascular changes.

BIOCHEMICAL INVESTIGATIONS

A specimen of urine (about 200 ml.) from the child was analysed by the Hingerty (1957) modification of the method described by von Euler and Floding (1955). This technique involves the extraction of the amines on alumina and treatment of the eluate from the latter with alkaline potassium ferricyanide in the presence of ascorbic acid. The trihydroxyindole derivatives so formed (Lund, 1952) exhibit strong green fluorescence under ultra-violet light and the intensity of this is compared with that produced by urine to which known additions of noradrenaline have been made.

Using this test the child's urine showed a fluorescence well in excess of that obtained from urine to which had been added 10 micrograms of noradrenaline per 100 ml.

urine. The presumptive diagnosis of phaeochromocytoma suggested by this result was thought by the clinicians to be unlikely and a sample of the alumina extract from the urine was examined by the Department of Physiology of Queen's University, Belfast, using a rat uterus assay preparation. The apparent catechol amine activity was less than 0·001 micrograms per ml. of the original urine, *i.e.*, less than 1/100 of the value obtained by the Hingerty method, and this was confirmed by tests on a further urine specimen from the patient using the rabbit blood pressure assay.

It is recognized that using the Hingerty method a positive result can occasionally be given by substances in the urine other than catechol amines (Frazer, 1957). Tetracycline (achromycin) has been incrimated in this respect (Glenn, 1959) and the fact that oxytetracycline (terramycin) was being given to our patient at the time of the initial catechol amine estimation suggested that this antibiotic might be the substance responsible for the observed false positive result. The fact that the catechol amine value had fallen to near normal levels when repeated one week after discontinuing antibiotic therapy gave extra support to this belief. Accordingly, the following experiment was set up *in vitro*. Noradrenaline, reserpine (serpasil), digoxin, mecamylamine (inversine), and oxytetracycline (terramycin) were added to separate urine specimens obtained from healthy individuals. These were then carried through the following biochemical extraction.

Each urine sample was evaporated under vacuum after bringing to *p*H 4 and the dried extracts treated with acid acetone. The insoluble matter was discarded and after evaporation to dryness the residue was again taken up in acid acetone. The evaporation was repeated and the final residue was dissolved in acetone and applied to paper strips for chromatography. This was carried out in the dark using the butanol-acetic-acid-water system. The dried strips were sprayed with potassium ferricyanide and alkaline ascorbic acid and examined under ultra-violet light for the appearance of fluorescence.

The urine samples containing noradrenaline and oxytetracycline each gave a bright fluorescence (Table I).

TABLE I

Substance Added to Urine	*Colour of Fluorescence under Ultraviolet Stimulus after Ferricyanide Oxidation*
Noradrenaline	Bright green
Reserpine	White
Digoxin	Non-specific
Mecamylamine	Non-specific
Oxytetracycline	Bright yellow-green

Since the estimation of catechol amines is based on the intensity of their fluorescence it is clear from these observations that false positive results could be obtained in patients on oxytetracycline should this antibiotic be excreted in the urine in sufficient concentration. To test this possibility catechol amine estimations were carried out by the Hingerty (1957) method on urine collected from 11 children who were confined to bed and receiving treatment with antibiotics of the tetracycline group. Specimens were collected one and seven days after starting treatment.

The results, which are set out in Table II, show in many instances a significant rise in the urine catechol amine levels (normal values for children = 40 μg./24 hours). There was no obvious relationship between the catechol amine values and either the dose, method of administration, or type of antibiotic tested. On the other hand, the values recorded after a seven-day period of treatment tended to be higher than those noted after only 24 hours of therapy.

TABLE II

Patient Number	*Antibiotic*	*Daily Dose (mg./kg. body weight)*	[1]*Catechol Amines as Noradrenaline in 24 Hours (μg.)*	
			After One Day	*After Seven Days*
1	Tetracycline	20	98	65
2	Tetracycline	14	54	106
3	Tetracycline	21	93	124
4	Chlortetracycline	21·5	83	102
5	Chlortetracycline	16	73	135
6	Chlortetracycline	11	106	104
7	Erythromycin	24	65	135
8	Erythromycin	27	53	79
9	Erythromycin	24	39	69
10	Oxytetracycline	22·5	86	57
11	Oxytetracycline	29	26	76

[1]Estimated by the method of Hingerty (1957)

DISCUSSION

The investigations described show that when estimated by the Hingerty method a high urine catechol amine value can be obtained if the patient is receiving treatment with one of the tetracycline group of antibiotics. Although raised, the levels noted in the group of children receiving treatment with antibiotics of the tetracycline group were not as high as the initial value recorded in the patient E.P. Two factors probably responsible for this discrepancy would seem to be the more prolonged period of treatment (two weeks) and the much higher dosage schedule used in the case of E.P. (47 mg./kg.). Nevertheless, the urine catechol amine values recorded were some two to three times in excess of the normal standards for children accepted in our laboratory using this method. Since results of this order have been noted in patients with phaeochromocytoma (McMillan, 1957), it is clearly essential that patients should be taken off all tetracycline antibiotics some days before collecting urine for the biochemical estimation of catechol amines.

We should like to thank Professor F. M. B. Allen, of the Department of Child Health, The Queen's University, Belfast, for his interest and encouragement, and Professor A. D. M. Greenfield, of the Department of Physiology, for the biological assays.

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J. clin. Path. (1961), **14**, 418

A sensitive method for the colorimetric determination of urea

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SYNOPSIS A method is described for the direct colorimetric determination of urea in biological fluids. The method depends on the reaction (first described by Wheatley, 1948) between urea, diacetylmonoxime, and phenylanthranilic acid in the presence of controlled amounts of oxidant; chloride ions are included to sensitize the reaction; manganous ions stabilize the resultant colour; and phosphate enables reasonable reproducibility to be achieved.

The method is rapid and suitable for routine analytical purposes. Precision and accuracy are good; sensitivity is high for an activated acid reagent up to about one week old, and thereafter decreases.

A hypothetical mechanism for colour formation is presented.

Of the numerous methods published for the direct colorimetric determination of urea and its derivatives, the most investigated have been those based on the Fearon (1939) carbamido reaction between the urea compound and diacetyl monoxime (DAM), *iso*-nitrosopropiophenone, or diacetyl (Ormsby, 1942; Barker, 1944; Archibald, 1945; Wheatley, 1948; Natelson, Scott, and Beffa, 1951; Friedman, 1953; Koritz and Cohen, 1954; Rosenthal, 1955; Kawerau, (1946); Marsh, Fingerhut, and Kirsch, 1957; Le Mar and Bootzin, 1957; Sardou, 1958; Girard and Dreux, 1958; Crokaert and Schram, 1958; Holden, 1959).

The number of attempts which have been made to meet the difficulties encountered is indicative of the magnitude of the problem. The disadvantages which have to be overcome include the instability of the resultant colour to light and time; the differential effect of light on blanks and test solutions; low to moderate sensitivity; prolonged heating time for reaction; sensitivity of colour intensity to reaction temperature; the objectionable nature of the reagents, *e.g.*, concentrated hydrochloric acid, arsenic compounds; lack of proportionality between urea concentration and colour over a sufficiently wide range.

The present work is an attempt to develop reagents which minimize or obviate these various disadvantages. A discussion of the chemistry of the reactions involved is given.

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METHOD

REAGENTS The following are required:—

(1) *Zinc sulphate-sodium sulphate isotonic solution* Dissolve 2·6 g. zinc sulphate ($ZnSO_4 \cdot 7H_2O$) A.R. and 12 g. sodium sulphate anhydrous (or 27 g. decahydrate) in water and dilute to 1,000 ml.

(2) *Sodium hydroxide, 0·5 M.*

(3) *Sulphuric acid, 50% v/v (9 M) A.R.*

(4) *Manganous chloride ($MnCl_2 \cdot 4H_2O$) A.R., 40% w/v.*

(5) *Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) A.R.*

(6) *Sodium nitrate A.R. 0·05 M* Dissolve 0·4250 g. solid in water and dilute to 100 ml. and refrigerate.

(7) *Hydrochloric acid concentrated A.R. (10 M)*

(8) *Diacetylmonoxime (DAM) 1% in 0·02% v/v acetic acid* Refrigerate.

(9) *Phenylanthranilic acid (PAA) 0·1% in 20% v/v ethanol-1% sodium bicarbonate A.R.* Dissolve 100 mg. PAA in 20 ml. pure ethanol, add 1 g. sodium bicarbonate and about 60 ml. water. Dissolve and dilute to 100 ml. and refrigerate.

(10) *Acid phosphate stock reagent* To 312 g. sodium dihydrogen phosphate in a 3-litre flask add about 250 ml. water, followed by 1,230 ml. sulphuric acid 50% v/v. Add 100 ml. manganous chloride 40% and 40 ml. 10 M hydrochloric acid. Mix well and dilute to 2,000 ml. This reagent keeps indefinitely.

(11) *Activated acid phosphate reagent* Measure 4·0 ml. sodium nitrate 0·05 M into a 1,000 ml. flask; add rapidly 500 ml. acid phosphate stock reagent (10). Mix well and allow to stand at room temperature for 17 to 24 hours before setting up a calibration, although the reagent may be used immediately if standards and a blank are included with the test. This reagent keeps for about one week at

room temperature. Check regularly with the standards.

(12) *DAM—PAA reagent* Mix equal volumes of reagents (8) and (9). This reagent is best made in small quantities but keeps for several days in the refrigerator. The separate reagents appear to be stable for long periods, especially if refrigerated.

PROCEDURE FOR BLOOD SPECIMENS Measure 0·05 ml. blood, serum, or plasma into a tube containing 3·85 ml. reagent (1). Mix and add 0·1 ml. reagent (2). Mix and allow to stand for 2 min. Centrifuge or filter.

Measure 0·6 ml. protein-free solution into a Pyrex test-tube (6 in. × $\frac{5}{8}$ in.). Add 0·6 ml. reagent (12) and then run 5 ml. reagent (11) directly into the tube. The last one or two millilitres may be used to wash down the wall of the tube. Mix thoroughly. A purple colour appears if reagent (11) has matured for at least 15 hours.

Heat the tubes for 11 to 12 minutes in a bath made of metal and containing water boiling within the range 99 to 100·5°C. and equipped with an accurate thermometer. The purple colour disappears after about one minute's heating and a magenta colour slowly develops. After heating, cool immediately in running water to room temperature. Read in a spectrophotometer at 535 mμ or in a colorimeter using a yellow-green filter such as the Ilford filter No. 625.

PROCEDURE FOR URINE SPECIMENS The procedure is very simple. Dilute the urine 1 in 500 and 1 in 1,000. Take 0·6 ml. of each liquid and proceed as for blood analysis. At such high dilutions any interferences are usually of negligible proportions.

CALIBRATION Prepare a stock standard containing 62·5 mg. % urea A.R. Dilute 1, 2, and 3 ml. of this to 100 ml. These standards correspond to bloods containing 50, 100, and 150 mg. % urea. Take 0·6 ml. of each standard (containing 3·75, 7·5, and 11·25 μg. urea) and proceed as for blood specimens. Carry out duplicate determinations. Measure against a water blank, although, if preferred, a reagent blank may be used. Thereafter check the reagent daily by including one standard in duplicate.

EXPERIMENTAL

PRELIMINARY VARIATIONS IN ACID REAGENT COMPOSITION Initially, many combinations of sulphuric, hydrochloric, phosphoric, perchloric, arsenic, and other acids were investigated in conjunction with a DAM—PAA reagent as first employed by Wheatley (1948). The reagents used fell into the following groups, namely, those which (*a*) produced colour which was proportional over too narrow a range of urea concentration; (*b*) lacked sensitivity; (*c*) lacked stability either of the reagent or of the colour produced; (*d*) gave erratic results; (*e*) caused precipitation.

Combinations of sulphuric, arsenic, and moderate amounts of hydrochloric acid were found to give the best results. Marsh *et al.* (1957) found that 15% sodium chloride in their sulphuric-arsenic acid reagent increased sensitivity but led to loss of proportionality between urea concentration and colour. Rosenthal, in an excellent paper, showed that high concentrations of hydrochloric acid have a strongly inhibitory effect on colour formation from urea and DAM (PAA was not used by either of these workers.)

At a later stage of our work arsenic pentoxide was replaced by sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) for greater ease in preparing the reagents. Two grades of arsenate were used, one of A.R. purity and the other of laboratory reagent grade. Surprisingly, the A.R. material gave a reagent of poor sensitivity while the other was satisfactory. Analysis of the two grades showed that the main difference lay in the nitrate content. The A.R. material contained <0·001% while the other had a content of ~0·008% NO'_3. This implicated nitrate ion and not arsenate as the oxidant responsible for colour formation in our method. Omission of arsenate, but inclusion of small amounts of nitrate, gave good but erratic results, so it appeared that the arsenate had some stabilizing or 'buffering' effect. This suggested the use of other salts such as citrate, tartrate, and phosphate. Citrate and phosphate were both effective in producing intense and reproducible colours, but phosphate was finally selected since it is obtainable in a state of high purity. Phosphate-nitrate mixtures were found to give results as good as impure arsenate and were preferred since the amount of oxidant is controllable.

Other oxidants such as chlorate, perchlorate, bromate, iodate, and permanganate were investigated but proved unsatisfactory for various reasons.

Apart from PAA, other sensitizers were employed. These included other diphenylamine derivatives, quinones, and quinone imines. Of these only 4-aminodiphenylamine was found to be as effective as PAA, but since it is less stable, PAA was retained. Diphenylaminesulphonic acid (sodium salt) also produces good sensitivity but the concentration required is about four times that of PAA.

CERTAIN EFFECTS OF VARIATION IN THE REAGENTS FINALLY SELECTED When each constituent of the optimum reagents was varied in turn from the optimum used in the method, the following effects were produced:—

Acid phosphate reagent Reduction in molarity of the sulphuric acid below 5·5 M caused loss of sensitivity; above that level overoxidation of PAA, loss of hydrochloric acid, and marked turbidity were observed. Satisfactory results were obtained within the range 5 to 6 M.

The hydrochloric acid could be varied over the range 0·1 to 0·4 M without effect. Higher concentrations led to loss of colour and formation of a brown flocculent precipitate.

Increasing phosphate ion concentration up to 1M gave increasingly better reproducibility and colour stability but no change in sensitivity. At appreciably higher concentrations crystallization of salts is trouble-some.

Concentrations of manganese chloride below 0·1 to 0·2 M failed to produce satisfactory light- and time-stability. Higher concentrations yielded no further improvement.

The optimum range for nitrate ion concentration was found to be 0·32 to 0·48 mM. Higher concentrations produced higher blanks and eventually destruction of the colour formed. Sensitivity dropped rapidly below the 0·3 mM level.

Diacetylmonoxime The optimum range was found to be 0·8 to 1·2%. Below this range loss of sensitivity occurred and above it colour suppression was observed. Rosenthal (1955) found the same effects for his reagents. No special purification of the DAM was necessary.

Phenylanthranilic acid The optimum concentration range was 0·098 to 0·12%. Below this range there was loss of linearity at the higher urea levels and above it turbidities were produced. A slight opalescence is produced even within the optimum range but is not sufficiently serious to interfere with the colorimetric measurements.

Variation of relative volume of active acid reagent used in the test The ratio of the volume of active acid reagent to that of the remainder (1·2 ml.) could be varied over the range 6 : 1·2 to 3 : 1·2 without loss of sensitivity (after allowing for volume change). A ratio of 5 : 1·2 was chosen to give a convenient volume for colorimetric measurement.

TIME OF HEATING AT 100°C. Using the optimum reagents and the volumes described under Method, a series of urea standards was heated for periods ranging from 4 to 14 min. (Fig. 1).

The colour reaches a maximum in 12 min. and then begins to decrease, prolonged heating periods destroying the colour. A period of 11 to 12 min. is therefore suitable.

EFFECT OF REACTION TEMPERATURE ON COLOUR PRODUCTION Colour development was carried out over the range 90° to 105°C. in a bath controlled to ± 0·5°C. (Fig. 2). The optimum temperature range is 99° to 100·5°, so that a boiling water bath equipped with a calibrated thermometer is ideal under the usual conditions of atmospheric pressure.

A bath constructed of metal is preferrred since at 100°C. the coloured product is somewhat sensitive to light, values some 6% lower being observed when the reaction is carried out in daylight.

STABILIZATION OF COLOUR One of the main difficulties in the use of DAM methods (with or without the use of PAA) has been the sensitivity of the blanks and colours to light. This problem is more acute when sensitizers such as PAA are employed and this has probably been a significant reason for their avoidance.

In our search for suitable stabilizers a variety of metal ions was tried. Of these, only Sb^{3+}, Cu^{2+}, and Mn^{2+} were found to be effective. Manganese was selected because it contributes virtually nothing to the blank reading and is not toxic. With manganese chloride at the 0·2M level in the acid reagent it was found that both blanks and test colours could be irradiated at 20° to 25°C. in direct sunlight for 30 min. without significant change. In diffuse daylight or in darkness, at a level of 0·1M manganese chloride, the colour decreased by 5% per hour so that this concentration was considered adequate for normal working conditions.

SPECTRUM The absorption spectrum of the urea reaction product (curve 1, Fig. 3) has a broad band in the visible with $\lambda_{max.}$ at 535 mμ. The nature of the spectrum is such that accurate measurements may be made even with a colorimeter equipped with a moderately narrow band-pass filter.

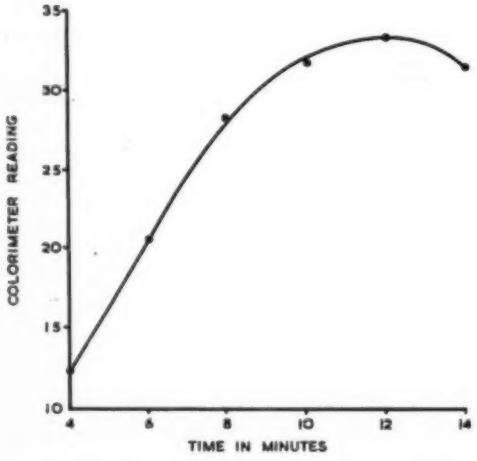


FIG. 1. *Effect of time of heating at 100°C. on colour production.*

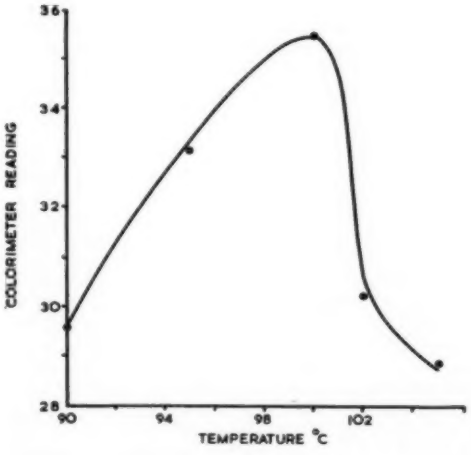


FIG. 2. *Effect of reaction temperature on colour production.*

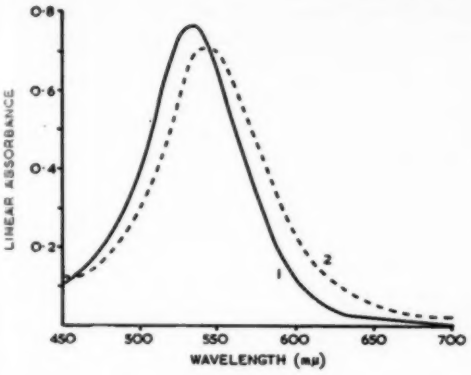


FIG. 3. *Curve 1: Absorption spectrum of reaction product from 0·25 μ mole urea.*
Curve 2: Absorption spectrum of reaction product from 0·25 μ mole phenyl urea.
The curves were measured on the Beckman DK2 spectrophotometer using 1 cm. cells.

RESULTS

COMPARISON OF PRESENT METHOD WITH A UREASE METHOD Comparisons were made over a period of time under ordinary routine conditions between the present method and a method employing urease and Nessler's reagent (Connerty, Briggs, and Eaton, 1955). The results are presented in Table I and indicate a high specificity for urea in the present method, the average difference between the two being less than 3%. Single determinations only were made by each method for most of the blood specimens examined. In cases where duplicate or triplicate determinations were made by each method, closer agreement between them was evident. No exhaustive investigation of possible interferences was therefore undertaken. This matter has been dealt with by previous workers (see, for example, Wheatley, 1948; Koritz and Cohen, 1954; Rosenthal, 1955). These authors have shown that for blood and urine the relative colour equivalents and concentrations of potential interferences are such that, in general, no significant contribution from them is likely to be encountered.

REPRODUCIBILITY Table II shows the variations encountered when repeated determinations were carried out on several standards, using two different batches of reagent and separate blanks. Under

TABLE II

REPRODUCIBILITY OF DETERMINATIONS

Reagent Batch No.	*Urea (mg.%)*	*Colorimeter Readings*	*Mean Reading*	*Readings as % Mean*
7(a)	40	0·25		92·6
		0·28	0·270	103·7
		0·28		103·7
5	40	0·28		98·9
		0·29	0·283	102·5
		0·28		98·9
7(a)	80	0·53		102·7
		0·52		100·8
		0·53	0·516	102·7
		0·49		95·0
		0·51		98·8
5	80	0·53		101·9
		0·50	0·520	96·2
		0·53		101·9
7(a)	100	0·61		101·1
		0·62	0·603	102·8
		0·58		96·2

$\sigma = \pm 3{\cdot}33\%$ $3\sigma = \pm 10\%$

TABLE I

COMPARISON OF THE PRESENT METHOD WITH ONE USING UREASE

Serum No.	*Urea (mg.%)*		*% Difference Present-Urease*	*Serum No.*	*Urea (mg.%)*		*% Difference Present-Urease*
	Present	*Urease*			*Present*	*Urease*	
1051	42	38	+10	1342	27	26	+ 4
1059	88	73	+20	1417	43	48	−10
1066	58	55	+ 6	1418	42	43	− 3
1067	26	26	0	1425	30	26	+16
1090	28	26	+ 8	1492	32	34	− 6
1310	25	28	−12	1493	63	63	0
1322	16	16	0	1601	56	56	0
1327	58	59	− 2	1602	31	30	+ 3
1328	68	76	−12	1604	38	38	0
1329	34	33	+ 3	1678	123	125	− 2
1331	40	34	+18	1608	69	63	+10
1332	33	32	+ 3	2237	424	381	+10

Average % difference + 2·7%

average conditions, and with urea levels as low as 40 mg. %, overall confidence limits of ± 10% cover all results including variations in the blanks (Henry and Segalove, 1952).

RECOVERIES FROM SERUM Varying amounts of standard urea were added to blood sera and the tests carried out in the usual way. The results are shown in Table III. All determinations were carried out in duplicate and the mean values taken. Overall recoveries thus appear to be satisfactory. All measurements were made using a Beckman DK2 or a Unicam SP600 spectrophotometer.

TABLE III

RECOVERIES FROM BLOOD SERA

Serum Urea (mg.%)	*Urea Added* (mg.%)	*Total Recovered* (mg.%)	*Added Urea Recovered* (mg.%)	*% Recovery*
43	20	64	21	105
	30	72	29	97
32	53·7	89	57	106
	107·4	137	105	98
53	100	151	98	98
	300	366	313	104

Mean recovery 101 ± 4%

CALIBRATIONS Three different instruments were used to measure a series of standards varying from 0 to 500 mg. % urea. The instruments were, in order of increasing order of optical resolution: (*a*) the Eel filter colorimeter, (*b*) the Unicam SP600 spectrophotometer, and (*c*) the Beckman DK2 ratio recording spectrophotometer.

Figure 4 shows typical calibration curves for these instruments. The results show clearly that there is a linear relationship between urea concentration and resultant colour up to a sample level of at least 20 μg. urea. The range of observed linearity for any particular instrument is determined by the degree of resolution obtainable, being much lower for a filter instrument than for one possessing a monochromator. A filter instrument is satisfactory up to a level of about 150 mg. %; for higher sample values it is probably best with this instrument to repeat the test using a smaller aliquot of protein-free filtrate. The Beckman DK2 spectrophotometer gave linear results up to about 30 μg. urea, corresponding to a blood urea level of at least 400 mg.%.

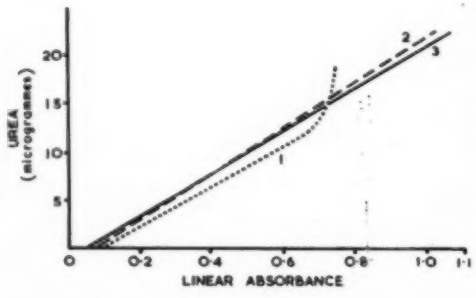


FIG. 4. *The relationship between the amount of urea and linear absorbance of the reaction product, when measured on three different instruments: 1 Eel colorimeter, 1·5 cm. diameter tubes; 2 Unicam SP600 spectrophotometer, 1 cm. cells; 3 Beckman DK2 ratio recorder, 1 cm. cells.*

DISCUSSION

In investigations of the Fearon reaction as applied to urea, many workers have included, as did Fearon, an oxidant, the purpose of which usually has been stated to be to destroy the hydroxylamine produced by hydrolysis of the DAM. However, Archibald (1945), using *iso*-nitrosopropiophenone, and Holden (1959) both omit oxidant from their reagents and obtain reasonable sensitivity. We find that somewhat more of the yellow product of reaction between urea and DAM is formed with our unactivated acid reagent than with the activated one. Furthermore, the addition of moderate amounts of hydroxylamine has no effect on the sensitivity of the reaction either in the presence or absence of oxidant. It appears therefore that not only is oxidant unnecessary in the Fearon reaction for urea but also that it is somewhat deleterious.

It has been assumed also that diacetyl liberated by acid hydrolysis is the compound which reacts with the urea (Fearon called the reaction the 'carbamide-biacetyl reaction'). Indeed, under the conditions of our test, it may be shown spectroscopically that approximately 90% of the DAM is hydrolysed in ten minutes. However, if the DAM is replaced by diacetyl in varying concentrations, much less colour formation is evident; the addition of hydroxylamine actually enhances colour formation, but the combination is still much less effective than DAM. This compound, and not diacetyl, would appear therefore to be the one which reacts with the urea. This would also account for the fact that a relative excess of DAM is required for optimum colour formation.

Little appears to be known concerning the nature of the reaction between urea and DAM. The possibilities which suggest themselves are: (1) Straight chain condensation between one molecule of DAM and one molecule of urea; (2) the same, with a 2 : 1 ratio of DAM to urea; (3) a 1 : 1 ring condensation with formation of a substituted 1, 2, 4-triazine.

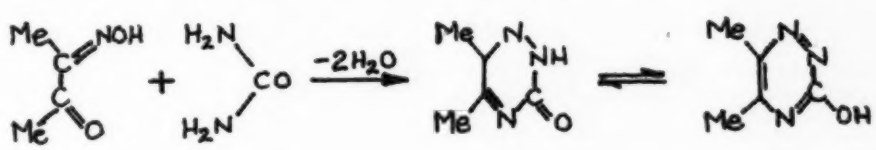
These reactions may be formulated thus:

(i) $Me.CO.C(Me):NOH + H_2N.CO.NH_2 \xrightarrow{-H_2O} Me.CO.C.(Me) : N.NH.CO.NH_2$

(ii) as (i) followed by condensation with a further molecule of DAM to give

$Me.CO.C(Me) : N.NH.CO.NH.N : C(Me).CO.Me$

(iii)



3-hydroxy-5, 6, dimethyl-1, 2, 4-triazine

with possibly the addition of one or more protons to the triazine ring under the strongly acid conditions used.

Evidence for one or another of these possible mechanisms was sought by studying the reactions between mono- or disubstituted ureas and DAM (using colour formation as a criterion of reaction). Table IV shows the results which might be expected for the three types of reaction outlined above, according to the nature of the urea derivative used.

Monophenyl urea reacted to give approximately the same amount of colour as an equimolar amount of urea (Fig. 3, curve 2), the absorption band being shifted bathochromically by some 10 mμ. *sym*-Diphenyl urea failed to produce colour.

No account has been taken of possible steric or deactivating effects which could result upon substitution but as far as it goes the evidence indicates some kind of ring, rather than chain condensation. The formation of the triazine derivative is suggested as a tentative hypothesis on the basis of which no reaction would be expected to occur with *asym.* disubstituted ureas.

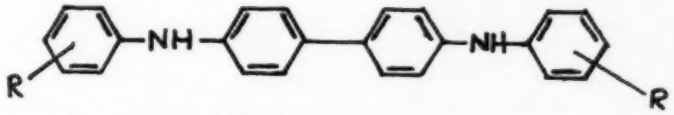
As mentioned earlier, numerous compounds were investigated as colour intensifiers and of these diphenylamine derivatives gave the most satisfactory results. This might indicate that the function of nitrate (acting either directly, or indirectly by oxidation of hydrochloric acid) in the acid medium is to oxidize the diphenylamines to diphenylbenzidines:

TABLE IV

POSSIBILITY OF REACTION BETWEEN SUBSTITUTED UREAS AND DAM

Urea Derivative	*Nature of Reaction*		
	One-step Chain	*Two-step Chain*	*Ring Formation*
Urea	+	+	+
Mono-substituted	+	+	+
sym. disubstituted	+	+	−
asym. disubstituted	+	−	−

This is perhaps reasonable in that at higher nitrate and acid concentrations a 'diphenylamine blue' is formed almost immediately, but under the conditions used here, the oxidation apparently stops at the intermediate diphenylbenzidine stage. Confirmation of this suggestion was sought first by carrying out the reaction using N,N′-diphenylbenzidine and unactivated acid reagent; the formation of a magenta colour indicated that the reaction proceeded in the manner suggested. Secondly, PAA in 85% sulphuric acid was oxidized with sufficient nitrate to convert it to the intensely coloured diphenylamine blue derivative. The warm solution was then reduced to the dicarboxy derivative of N,N′-diphenylbenzidine by the dropwise addition of sodium dithionite until the colour was just discharged. Concomitantly any excess nitrate was reduced. The reaction with urea was carried out using this reduced solution and unactivated acid reagent. Again, a magenta coloured product resulted, more intense than that obtained with an equivalent amount of diphenylbenzidine. The comparative behaviour of these two benzidine derivatives as regards intensity of colour production thus parallels the behaviour of PAA and diphenylamine (or diphenylamine sulphonic acid) on oxidation and thus suggests strongly that it is the diphenylben-



where R = 4-NH_2, 2-COOH or 4-SO_3H.

zidine structure which interacts with the urea-DAM condensation product.

Since it is difficult to conceive of a ready mechanism whereby this product could react with diphenylbenzidines, it is suggested tentatively that the final step in colour production is the formation of a magenta-coloured molecular complex from the two components, stabilized by manganese ions.

We wish to thank Dr. T. Orban of Sydney for his kindness in supplying notes of his (unpublished) modifications of Wheatley's method.

Approval has been given by the Director-General, Department of Public Health, for the publication of this work.

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The use of tetramethylammonium hydroxide in the Zimmermann reaction

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SYNOPSIS The use of tetramethylammonium hydroxide (T.M.A.H.) in place of potassium hydroxide in the Zimmermann reaction has been investigated. Although various pure steroids have different colour equivalents, a comparison of the results of 17-ketosteroid and 17-hydroxycorticosteroid estimations on a series of urines showed that the differences for the two reagents were only small and may be ignored. Tetramethylammonium hydroxide has the considerable advantage over potassium hydroxide of stability and need not be prepared freshly. The correction procedures available are discussed.

The reaction described by Zimmermann (1935) is now widely used for the estimation of 17-ketosteroids, particularly in crude urine extracts, and depends upon the violet colour developed by mixing solutions of potassium hydroxide, dinitro-benzene, and ketosteroid. A careful study of the reaction was made by Callow, Callow, and Emmens (1938), and subsequently many modifications have been proposed with the object of simplifying the procedure or rendering it more specific. The main disadvantages of its use are that different steroids have different colour equivalents, and when used for crude biological extracts, materials other than steroids react to produce a non-specific interference. Wilson (1954) has suggested carrying out the reaction at a lower temperature to give more nearly equal colour equivalents, and various formulae have been proposed to correct for the interfering chromogens by the use of extinctions at two or three wavelengths. Alternatively, the chromogen may be extracted with organic solvent, which leaves a considerable proportion of the interfering material in the residual solution. Most of these modifications have retained the use of potassium hydroxide in the reaction, but more recently, Bongiovanni, Eberlein, and Thomas (1957) and Glenn (1959) have proposed the use of organic base instead, and it was of interest to w if this offered any advantage in the estimation etosteroids. This paper reports some investi- ons made using tetramethylammonium hydroxide (T.M.A.H.), which is easily available, together with correction procedures which involve a minimum of labour.

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MATERIALS AND METHODS

m-DINITROBENZENE British Drug Houses, of a quality 'specially purified for the determination of 17-ketosteroids', dissolved in ethanol.

POTASSIUM HYDROXIDE AND SODIUM HYDROXIDE PELLETS Hopkin and Williams Analar.

ETHANOL was refluxed for two hours with 5 g./litre of dinitrophenylhydrazine and 10 ml./litre of concentrated hydrochloric acid, and distilled twice through a column.

ETHER (May and Baker) was used without purification.

TETRAMETHYLAMMONIUM HYDROXIDE Twenty-five per cent w/w aqueous, Hopkin and Williams.

ETHYLENE DICHLORIDE (British Drug Houses) was used without purification.

ETHANOLIC POTASSIUM HYDROXIDE 2·5 N was prepared as described by Wilson (1954) and stored under nitrogen in the refrigerator.

STEROIDS Solutions were prepared by weighing the steroids on a micro-balance and dissolving them in ethanol to give a concentration of approximately 50 mg./100 ml.; these solutions were kept in the refrigerator in stoppered tubes and allowed to warm to room temperature before use. For urine estimations a standard solution of dehydro*epi*androsterone (50 mg./100 ml.) was used. Twenty-four-hour urines were collected under toluene

(10 ml.) and stored at 4° until used. The volume was adjusted to 2,000 ml. with distilled water.

STOPPERED TUBES 'Excelo' 50 ml., with B.24 standard joint; 15 ml. with B.14 standard joint and calibrated to 10 ml. in 0·2 ml. divisions.

ANTI-BUMPING GRANULES British Drug Houses.

METHOD OF ESTIMATING URINARY 17-KETOSTEROIDS The urine was well shaken and two 10 ml. aliquots were pipetted into stoppered 50 ml. tubes. Then 3 ml. of concentrated hydrochloric acid was added and the tubes were shaken and placed in a boiling water bath for 10 min. They were then transferred to a cold water bath and when cool, 10 ml. of ethylene dichloride was added, the tubes stoppered and agitated for 15 min. in a horizontal shaker. The tubes were centrifuged for 10 min. at 2,000 r.p.m. and the urine layer removed by suction. The solvent was then filtered through a No. 1 Whatman filter paper into a clean stoppered 50 ml. tube and approximately 20 pellets of sodium hydroxide added. The tubes were stoppered and again shaken for 15 min. The solution was then filtered through Whatman No. 1 filter paper into calibrated tubes, and the volume reduced to 7 ml. with a fine pipette. A few anti-bumping granules were added and the tubes placed in a water bath at 90° in a fume cupboard, until the solvent had evaporated. The residue was finally dried by brief application of a vacuum or nitrogen, and the extract dissolved in 0·2 ml. of ethanol.

COLORIMETRY Colorimetry was carried out in potassium hydroxide and in T.M.A.H.

Potassium Hydroxide Five tubes were used containing 0, 0·05, 0·10, 0·15, and 0·2 ml. of the standard steroid solution. The volumes were adjusted to 0·2 ml. with ethanol. To each tube, including the urine extracts, was added 0·4 ml. of a 1 : 1 mixture of 2% dinitrobenzene and potassium hydroxide solution. The tubes were then stoppered, the contents mixed and kept at 25° in the dark for one hour. After this time, 2 ml. of aqueous ethanol (30% ethanol in water) was added, followed by 5 ml. of ether. The tubes were then shaken vigorously for 15 sec., and the ether layer decanted into a covered cuvette (1 cm. light path). Tests and standards were read at 515 mμ in the Unicam SP 600 against the blank tube. A calibration curve was then drawn and used to calculate the amount of steroid in the urine extracts. If a urine blank correction was to be made, one of the two extracts was used for this purpose, and was dissolved in 0·4 ml. of ethanol instead of 0·2 ml. A tube containing 0·4 ml. of ethanol was used as a reagent blank, and 0·2 ml. of potassium hydroxide solution was added to all tubes. After incubation and extraction as above, the urine blanks were read at 515 mμ against the reagent blank The optical density obtained was deducted from that of the appropriate test, and this corrected figure was used to calculate the amount of steroid present.

Tetramethylammonium hydroxide The procedure followed was exactly as described above, except that various concentrations of T.M.A.H. were substituted for the 2·5 N potassium hydroxide, and the alkali and 1% or 2% dinitrobenzene solutions were added separately, or mixed before addition, depending on the concentration used.

17-HYDROXYCORTICOSTEROIDS These were estimated as described by Appleby, Gibson, Norymberski, and Stubbs (1955), using 5 ml. of urine. Colorimetry was carried out as described above.

RESULTS

EXTINCTION MAXIMUM Using potassium hydroxide solution, the absorption maximum of the colour developed with dinitrobenzene and steroid was at 515 mμ in alcohol or ether. For the six ketosteroids examined, this was unaltered by substituting T.M.A.H., and all optical densities were therefore read at this wavelength. Fig. 1 shows calibration curves for dehydro*epi*androsterone using potassium hydroxide (2·5 N) and T.M.A.H. (2·7 N), and these are seen to be linear up to at least 100 μg. of steroid; using T.M.A.H., however, the colour developed by the alcohol blank was considerably lower. Furthermore, the colour developed by the T.M.A.H. for identical weights of steroid was 34% greater than with potassium hydroxide.

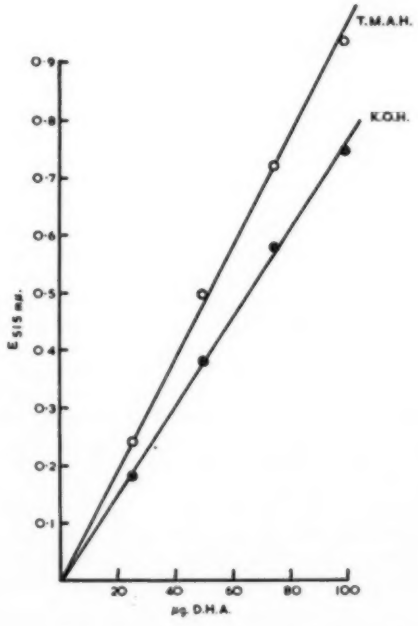

FIG. 1. *Calibration curves for dehydroepiandrosterone using KOH (2·5 N) and T.M.A.H. (2·7 N).*

STABILITY To ascertain the stability of the colour developed, readings were taken immediately after extraction of the colour, one hour later and at 17 hours. The results are shown in Table I, and indicate that after one hour the optical density has decreased by 2%, and fades linearly at 17 hours. Since readings are invariably made in under one hour, even with a large batch, this may be considered insignificant.

TABLE I

OPTICAL DENSITY OF ETHER SOLUTION OF ZIMMERMANN COMPLEX AT VARIOUS TIMES AFTER EXTRACTION

	Optical Density (515 mμ)
Immediate extraction	0·463
1 hour	0·454
17 hours	0·314

EFFECT OF CONCENTRATION OF T.M.A.H. The amount of colour developed at a given time and temperature depends upon the concentration of alkali. Fig. 2 shows standard curves obtained with T.M.A.H. at various concentrations, and demonstrates a progressive increase in colour with concentration. At lower alkali strengths, the curves become non-linear, due to the relatively lower amount of colour developed by the higher standards. Concentrations greater than 25% have not been studied, but the increase from 20 to 25% produced only a small change in the slope of the curve and 25% may well be near the maximum possible under these conditions.

EFFECT OF CONCENTRATION OF DINITROBENZENE The effect of concentration of dinitrobenzene at an alkali concentration of 25% is shown in Fig. 3. Below 1%, there is a sharp fall in the amount of colour developed, but from 1% to 2% this is practically unchanged. Calculation shows that the amount of dinitrobenzene present at a concentration of 1% represents an excess of 32 moles for 100 μg. of steroid. However, the nature of the reaction is as yet not understood, although it has been postulated by Zimmermann (1944) that the chromogen contains 1 mole of steroid to 1 of dinitrobenzene. The use of 1% dinitrobenzene would therefore appear to give maximum colour formation, and a linear calibration curve up to at least 100 μg. of steroid. It is advantageous to use this concentration since it can be

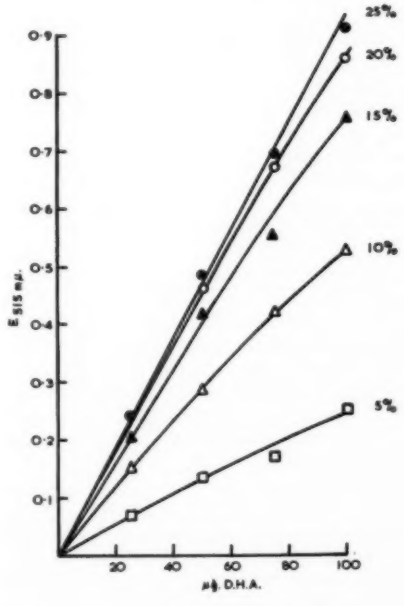

FIG. 2. *Standard curves for dehydroepiandrosterone at different concentrations of T.M.A.H. (2% dinitrobenzene).*

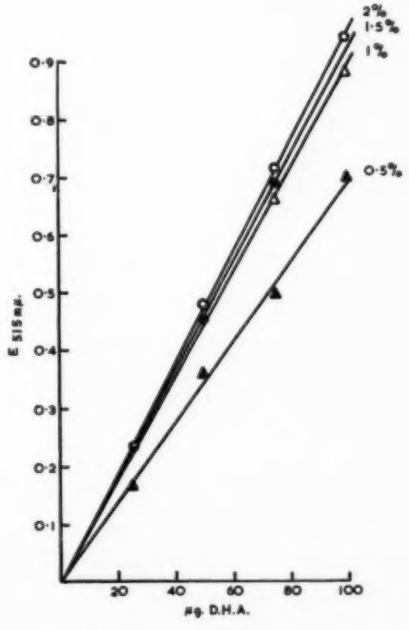

FIG. 3. *Standard curves for dehydroepiandrosterone at different concentrations of dinitrobenzene (2·7 N T.M.A.H.).*

mixed with 25% T.M.A.H. and added directly to the steroid solution, whereas at higher concentrations of dinitrobenzene, precipitation occurs, and it is necessary to add the reagents separately.

TIME OF DEVELOPMENT Fig. 4 shows the development of colour with time at room temperature and at 25°. In both cases the optical density increases only slowly after 60 min., although to obtain maximum colour it appears necessary to incubate for two hours. However, for routine estimations, a period of 60 min. is convenient and produces a large proportion of the total colour which can be developed. Also, after ether extraction at 60 min., the optical density alters by less than 2% up to one hour; it is necessary to use glass caps for the cuvettes to prevent evaporation of the ether.

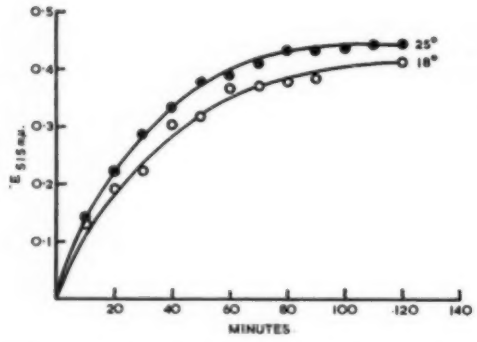

FIG. 4. *Development of Zimmermann colour with time at 18° and 25°.*

SENSITIVITY For the estimation of smaller quantities of steroid, it is convenient to employ half quantities of reagents. This gives a final volume of ether of 2·5 ml. and 5μg. of dehydro*epi*androsterone produces an optical density of 0·100. Quantities of steroid less than 5 μg. can be estimated with some accuracy, and the use of micro-cells would offer even greater sensitivity.

COMPARISON OF COLOUR EQUIVALENTS OF DIFFERENT KETOSTEROIDS, PURE STEROIDS, AND URINE EXTRACTS Different ketosteroids, such as those normally excreted in human urine, are chromogenic to different degrees in the Zimmermann reaction, and a knowledge of these colour equivalents is necessary to calculate absolute amounts of steroid from one standard. Alternatively a different standard may be required in each case. The colour equivalents will vary with different conditions of development, and a comparison was therefore made of the optical densities obtained by using potassium hydroxide and T.M.A.H. for pure reference steroids. The results are shown in Table II and are relative to dehydro*epi*androsterone. It will be seen that quite different

TABLE II

ZIMMERMANN COLOUR EQUIVALENTS OF EQUAL WEIGHTS OF VARIOUS 17-KETOSTEROIDS

Steroid	*DHA Colour Equivalent*	
	T.M.A.H.	*Potassium Hydroxide*
Dehydro*epi*androsterone	100	100
Aetiocholanolone	97	108
Androsterone	98	99
11β-Hydroxyaetiocholanolone	74	96
11β-Hydroxyandrosterone	65	73
11-Ketoaetiocholanolone	113	122

relative results were obtained using T.M.A.H. and potassium hydroxide, and it might therefore be anticipated that a difference would be found for the crude mixture of steroids obtained from human urine. A similar comparison was therefore made of 12 ketosteroid extracts obtained from normal urines. Table III shows these results, and in general it

TABLE III

17-KETOSTEROID CONTENT (MG./24 HR.) OF 12 NORMAL URINES USING KOH AND T.M.A.H.

	2·5 N KOH	*25% T.M.A.H.*	Δ
1	8·8	8·6	−0·2
2	2·9	2·4	−0·5
3	3·0	4·1	+1·1
4	5·5	5·5	0
5	2·0	2·5	+0·5
6	8·8	8·4	−0·4
7	4·2	4·3	+0·1
8	5·9	5·6	−0·3
9	3·7	3·9	+0·2
10	5·2	4·5	−0·7
11	9·8	8·5	−1·3
12	12·3	11·0	−1·3

appears that T.M.A.H. gives slightly lower values than potassium hydroxide, although this is not invariably true, and the difference is small. Similar results (Table IV) were obtained for 17-hydroxycorticosteroids estimated on 12 urines from patients with various disorders.

STABILITY OF T.M.A.H To investigate the stability of the T.M.A.H., a comparison was made of two batches, one obtained freshly from the manufacturers, and a second which had been already used and kept in the laboratory for one year. There was no significant difference in the calibration curves obtained or in the low reagent blanks; this is in

TABLE IV

17-HYDROXYCORTICOSTEROID CONTENT (MG./24 HR.) OF 12 URINES USING KOH AND T.M.A.H.

	2·5 N KOH	*25% T.M.A.H.*	Δ
	(*a*)	(*b*)	(*b*−*a*)
1	23·9	24·2	+0·3
2	32·8	31·4	−1·4
3	30·0	31·1	+1·1
4	31·4	30·1	−1·3
5	34·5	37·1	+2·6
6	27·6	27·2	−0·4
7	9·5	10·6	+1·1
8	12·3	9·9	−2·4
9	19·4	19·2	−0·2
10	15·7	16·1	+0·4
11	13·6	14·5	+0·9
12	15·0	15·3	+0·3

striking contrast to potassium hydroxide solution, which, however carefully preserved, will quickly become unusable, since the reagent blank increases rapidly.

DISCUSSION

A major source of error in the estimation of urinary ketosteroids is the presence of pigment and other material which contribute colour to that produced by the Zimmermann reaction. In general, two methods for obviating this error are in use. The first involves the application of a mathematical correction, and the equation proposed by Talbot, Berman, and MacLachlan (1942) has been widely used. Alternatively the formula deduced by Allen (1950) may be applied from optical densities obtained at three wavelengths. Both these formulae make the basic assumption that the interfering chromogens behave in a consistent and known fashion, and this is difficult to ascertain for individual extracts and may not be true. In the estimation of 17-hydroxycorticosteroids, the chemical manipulations involved are likely to alter the composition of the non-steroidal material, and the application of correction formulae would not be justified without prior investigation of the spectral characteristics. Braunsberg (1957) and later O'Sullivan (1958) devised tests for justifying the application of the Allen correction to crude extracts and such tests should undoubtedly be made before a correction may be considered valid. In any event, the time required to obtain further data for correction and their application will lengthen the time required for assay.

The second group of methods has the object of removing the interfering chromogens either before or after colorimetry, and the separation of ketonic and non-ketonic components by Girard's method is of this type. This is a lengthy process, requiring careful manipulation for quantitative results. and is not entirely suitable for routine estimations. Furthermore, the process often fails to eliminate non-steroidal 'ketonic' material. A different process was adopted by Drekter, Heisler, Scism, Stern, Pearson, and McGavack (1952) who used sodium hydroxide pellets to adsorb interfering pigments, and showed that unlike some other adsorbents, *e.g.*, charcoal, this material did not alter the recovery of ketosteroid. Alternatively, it is possible to purify the chromogen developed in the Zimmermann reaction by partitioning between an organic solvent and aqueous alcohol (Cahen and Salter, 1944; Henry and Thevenet, 1951; Zimmermann, Anton, and Pontius, 1952; Masuda and Thuline, 1953; Werbin and Ong, 1954; Crépy, Meslin, and Desgrez, 1956) and much yellow-brown pigment is then left in the aqueous layer of the reaction mixture. This component occurs even with pure ketosteroids, and is greatly increased with urine extracts. The colour due to remaining non-steroidal material is corrected for by the use of a parallel extract developed without dinitrobenzene. In our experience, these procedures are highly satisfactory, and treatment with sodium hydroxide pellets is often sufficiently effective to obviate the need for a urine blank when combined with solvent extraction of the Zimmermann chromogen. However, this blank correction is somewhat variable, and, where considerable quantities of pigment appear after hydrolysis, they may account for approximately 10% of the uncorrected optical density. It is preferable to adopt this correction routinely, otherwise some slight overestimate may be obtained. In the estimation of 17-hydroxycorticosteroids, the problem of interfering material is less important, since the preliminary reduction-oxidation process appears to alter the pigments chemically and later interference is small. Examination of the results from a series of urines from patients showed that the blank correction was negligible, and it was not considered necessary to adopt this for routine estimations[1].

At present, it is not easy to assess the extent to which any method for estimation of urinary ketosteroids approximates the true value, although purification by Girard separation is most likely to give fractions of reasonable purity. It has been shown that ether extraction of the Zimmermann complex gives values for urines which are in close agreement

[1]It should be noted that the ether extraction of the Zimmermann chromogens is less effective with polyketonic steroids. Thus the derivative of androstan-3, 11, 17-trione is only poorly extracted under these conditions. It has been observed (M.I. Stern, personal communication) that if the original ketogenic procedure is adopted for urine estimations, in which prior reduction with borohydride is omitted, the Zimmermann chromogens obtained are not extracted completely. This is probably due to the formation of polyketonic steroids in the oxidation procedure which are not formed when borohydride is employed.

with those obtained by Girard purification, whilst mathematical corrections still produce higher results (Zimmermann *et al.*, 1952). In the present investigation, urines were examined from a series of 20 women, of ages 18 to 47; the range of steroid excretion found was 4·7 to 16·8 mg./24 hr. with a mean of 11·3. The same urines were fractionated by enzyme hydrolysis and paper chromatography (James, 1961) into the component ketosteroids, and the total obtained was expressed as a percentage of the total estimated as described. This percentage varied from 50 to 108 with a mean of 70. Whilst this would appear to indicate that the crude method produces an overestimate, it should be borne in mind that the acid hydrolysis used will alter most of the naturally occurring steroids, which will then be estimated as various steroid compounds of which the colour equivalents may be highly variable. Thus, the total estimated will vary with the composition and will not necessarily bear any strict relationship to the total originally present and isolated by milder procedures. Any method employing vigorous hydrolysis will therefore produce results which at best are only indicative of the total steroid originally present, and if more accurate information is required, it is essential to employ more gentle methods. For routine clinical purposes, it is usually sufficient to obtain an indication of the total produced, and simple methods are therefore adequate.

Regarding the use of T.M.A.H. in place of the conventional potassium hydroxide in the Zimmermann reaction, it is clear that this introduces no gross error into the estimation, and it may therefore be considered as a suitable substitute. It has the advantage of producing more colour at the same normality, and this would give somewhat greater sensitivity. If pure steroid fractions are being estimated, it is necessary to remember that the colour equivalents of the various ketosteroids are different with the two reagents.

For routine ketosteroid and 17-hydroxycorticosteroid estimations, T.M.A.H. offers the considerable advantage of stability and even after one year at room temperature in the laboratory no detectable change in reagent blanks or calibration curve was observed. By contrast, potassium hydroxide rapidly becomes useless, since it produces increasing reagent blanks and has to be made freshly for each batch. Even when prepared as described by Wilson (1954) the reagent will keep only one month, is tedious to make, requires refrigeration, and has to be kept carefully under nitrogen. Tetramethylammonium hydroxide therefore appears to be well suited for use in a laboratory which undertakes only occasional steroid estimations, where the preparation of a large quantity of stabilized reagent is uneconomical.

We are indebted to Dr. W. Klyne for gifts of steroids from the M.R.C. reference collection, to Mr. J. Townsend for assistance with some of the estimations, and to Professor A. Neuberger, F.R.S., for his interest in this work.

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Laboratory studies with the systemic trichomonacide, metronidazole

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SYNOPSIS Laboratory studies with the trichomonacidal agent, metronidazole, show that 66 strains of *T. vaginalis* were killed in three days by concentrations of 0·0625 μg./ml. to 1 μg./ml.

Estimations of serum and urine levels in nine volunteers, the serum levels in 31 women, and the urine levels in 33 women show that metronidazole is rapidly absorbed and excreted in high concentration in the urine.

Development of resistance to metronidazole by *T. vaginalis* has not been demonstrated either *in vivo* or *in vitro*.

No toxic effect was demonstrated in the blood, liver, or kidney.

For some time it has been apparent that the effective treatment of infections due to *T. vaginalis* lies in a systematically administered drug. Aminitrazole was introduced in America (Plentl, Gray, Neslen, and Dalali, 1956) but whereas in common with many other compounds it showed considerable activity *in vitro*, it failed to produce blood levels and its use has been discontinued following unsuccessful trials (Catterall and Nicol, 1957; Dunlop, Philipp, and Watt, 1958; Jennison, 1957; Willcox, 1957).

In France, Cosar and Julou (1959) investigated metronidazole and found that it had a high anti-trichomonal activity. Durel *et al.* (1960) showed that effective blood concentrations of this drug were obtained after oral administration and correlated this with cure of trichomoniasis and lack of toxicity. The efficacy of metronidazole has since been confirmed in Great Britain (Moffett and McGill, 1960; Rees, 1960; Watt and Jennison, 1960a).

Metronidazole has the formula 1-beta-hydroxyethyl-2-methyl-5-nitroimidazole. Its solubility in water is approximately 1%, and in ethanol, ether, and chloroform approximately 0·5%. The *p*H of a saturated aqueous solution at 20°C. is about 5·8. In the dosage used clinically it has no bactericidal activity and therefore does not interfere with normal vaginal flora. It has no antifungal activity.

The clinical trial of metronidazole reported by Watt and Jennison (1960a) continues, and the present paper describes some of the laboratory investigations carried out during that trial. These investigations were directed towards elucidating the following points: (1) The sensitivity *in vitro* of various strains of *T. vaginalis* to metronidazole; (2) the possible development of acquired resistance to the drug *in vitro* or *in vivo*; (3) the levels of metronidazole in the blood and urine after ingestion of single doses by volunteers and after treatment with the recommended total dosage; (4) the possibility of toxic effects of the drug on the blood, liver, and kidneys.

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MATERIAL AND METHODS

One hundred and eight women suffering from vaginal trichomoniasis have been treated with metronidazole as out-patients. The methods of diagnosis and tests of cure were as described in a previous paper (Watt and Jennison, 1960a) and the length of observation varied from one to 23 weeks, with 60 patients (55·5%) having been observed for 12 weeks or longer. All patients were treated with a standard course consisting of 600 mg. metronidazole daily for seven days. After treatment, 21 (19·4%) have shown recurrence of *T. vaginalis*, but of these only four (3·7%) showed *T. vaginalis* at the first examination immediately following the course of treatment. These four patients must be considered as failures. Eleven of the male consorts of these 21 patients have been examined and six (54·5%) have been found to harbour *T. vaginalis*. The method used for demonstrating *T. vaginalis* in the male has been reported elsewhere (Watt and Jennison, 1960b).

The medium used for isolation of *T. vaginalis* was described by Stenton (1957), except that if monilia were seen in direct examination nystatin was added to the

medium for primary culture. The same medium, omitting antibiotics in case of possible synergistic action, was used in the estimation of blood and urine levels of metronidazole and the sensitivity of *T. vaginalis* to that drug.

The stock strain of *T. vaginalis* used throughout these investigations had been isolated about seven months previously. This stock strain was maintained by subculturing 0·1 ml. of culture into 10 ml. of fresh medium every three days. It was considered that by subculturing frequently and using a measured inoculum of *T. vaginalis* standardization could be maintained throughout the investigation. The sensitivity of the stock strain was determined at first biweekly and later at weekly intervals. After 40 subcultures no change in sensitivity was noted.

Trichomonacidal levels of metronidazole for the various strains of *T. vaginalis* were estimated by subculturing 0·1 ml. of a three-day growth of culture into 10 ml. of Stenton's medium containing serial dilutions of metronidazole and incubating for three days at 37°C. The cultures were then examined microscopically for living protozoa. Serum and urine levels were similarly determined by inoculating the stock strain of *T. vaginalis* in the ratio of 1:100 into medium containing dilutions of serum and urine. By comparison with the sensitivity of the stock strain the amount of metronidazole in serum or urine could be estimated.

RESULTS OF INVESTIGATIONS

SENSITIVITY OF T. VAGINALIS TO METRONIDAZOLE Sixty-six strains of *T. vaginalis* were tested for sensitivity to metronidazole; 55 of these were from women before treatment, seven were from women who did not respond to treatment or in whom the infection recurred later, and four were strains from male consorts.

Trichomonacidal levels varied from 1 μg./ml. to 0·075 μg./ml. but 51 (77%) of the strains were sensitive in the range of 0·25 μg./ml. to 0·125 μg./ml. (Fig. 1).

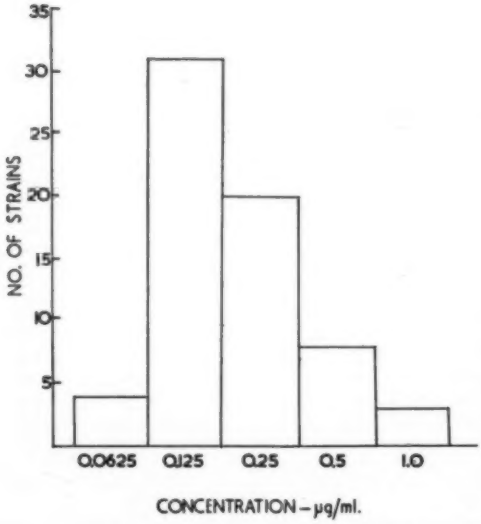

FIG. 1. *Trichomonacidal levels of metronidazole for 66 strains of* T. vaginalis.

ACQUIRED RESISTANCE The strains isolated from patients who did not respond to treatment did not show any increased resistance to the drug.

An attempt to induce resistance *in vitro* was made by repeated subcultures from the immediate sublethal dilution. It was found, however, that the sublethal dose was trichomonacidal when the organisms were exposed to it for longer than three days, *e.g.*, five days. Thus if cultures were incubated longer the organisms were sensitive to a lower concentration of metronidazole. Subcultures from tubes containing still lower concentrations of metronidazole also showed that prolonged exposure had a trichomonacidal effect. The method of subculturing from the immediate sublethal dilution is therefore not applicable in testing for the development of resistance to this drug *in vitro*.

SERUM AND URINE LEVELS These were estimated after a single dose and after a standard course.

After a single dose of 200 *mg.* A single dose of 200 mg. of metronidazole was given to nine volunteers and blood and urine samples collected at hourly intervals for four hours. In all subjects serum and urine after one hour showed trichomonacidal activity greatly in excess of the level required to inhibit the most resistant strain of *T. vaginalis* (Figs. 2 and 3). The peak was reached in most cases between two and three hours and thereafter the trichomonacidal activity diminished.

After a standard course of treatment All patients were treated as out-patients so it was impossible to collect specimens at a standard time. Specimens were collected when the patients attended on the last day of the course of treatment and the interval since taking the last 200 mg. tablet was recorded.

The serum and urine levels were estimated and are shown in Figs. 4 and 5. The levels obtained in patients who had not responded to treatment or who relapsed later are indicated. There is no evidence to suggest defective absorption of metronidazole in these patients.

In one patient who was lactating the level of metronidazole in the breast milk corresponded exactly to the serum level.

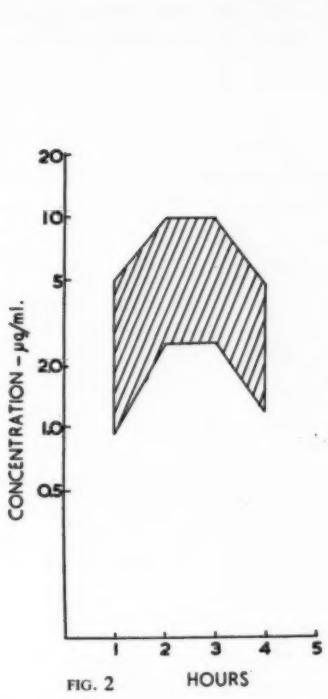

FIG. 2. *Composite graph drawn from estimations of trichomonacidal activity in the serum of nine normal subjects after the ingestion of 200 mg. of metronidazole.*

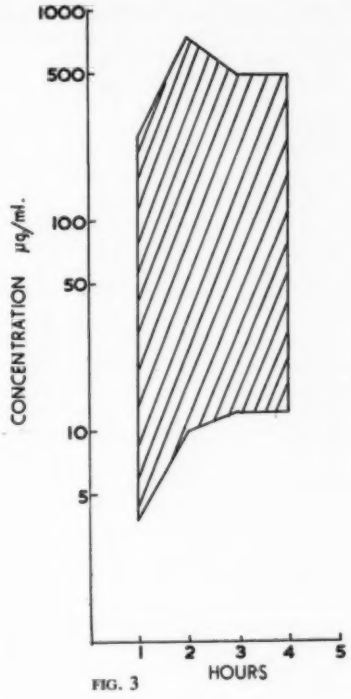

FIG. 3. *Composite graph drawn from estimations of trichomonacidal activity in the urine of nine normal subjects after the ingestion of 200 mg. of metronidazole.*

TOXIC EFFECTS Because of the nitro group in metronidazole the possibility of toxic effects in the haemopoietic system or liver was considered. The possibility of renal damage was also examined because of the high urinary concentration of the drug.

Haemoglobin estimations, red cell, total and differential white cell, and platelet counts were carried out before and after treatment in eight patients and at various times up to three months after treatment in 48 patients. No significant change in the blood picture was noted.

Serum alkaline phosphatase and serum glutamic pyruvic transaminase were estimated in 12 patients after completion of the course of treatment but no increase in values occurred.

In most patients the urine was examined for albumin, cells, and casts but no abnormality was detected.

DISCUSSION

Durel, Roiron, Siboulet, and Borel (1960), using the method described by Bushby and Copp (1955), found that the inhibitory level of metronidazole was 1 : 400,000 *in vitro*. This is based on the 99% death rate at 24 hours, and, using this method, we demonstrated inhibitory levels between 1 : 300,000 and 1 : 500,000 in six strains. But the method used throughout this investigation differs in that, since three days is required for maximum growth in the medium used, the concentration of the drug in which no growth had occurred at the end of three days' incubation was taken as the trichomonacidal level. This method also more closely simulates the conditions in a patient undergoing treatment, both as regards sensitivity of the strain of trichomonad and the blood levels. The longer incubation caused inhibition at concentrations which would have allowed growth after 24 hours and by this method trichomonacidal levels have been found to vary from 1 : 1,000,000 to 1 : 13,300,000 (1µg./ml. to 0·075 µg./ml.).

The inverse relationship between time and drug concentration was also apparent in the experiments designed to induce resistance *in vitro*. This is the converse of the effect of nystatin on *C. albicans* where the yeasts grow in higher concentrations of the antibiotic following prolonged incubation (Jennison and Stenton, 1957).

The blood and urine estimations show that metronidazole is rapidly absorbed and rapidly excreted and concentrated by the kidney. The blood levels are maintained at an adequate level for some hours. Comparison of the blood and urine levels with the strain sensitivities shows that even the most resistant trichomonad should have been eliminated in every patient. At least five times the trichomonacidal level was maintained in the blood during the 12 hours after treatment ceased, and in the urine the lowest ratio in an individual patient was 200 : 1. It seems therefore that in those patients who did not respond to treatment this was due to failure of metronidazole to reach a nidus of infection rather than to a resistant strain or to failure to absorb the drug. The experimental relationship between concentration of metronidazole and time of incubation suggests that prolonging the course of treatment rather than increasing the dose might be more successful in these patients. Reinfection probably plays an important part in late recurrences. All except one of the strains isolated from consorts had the same drug sensitivity as that from his female partner. The one strain with a different sensitivity was in fact more sensitive, suggesting the existence of separate strains.

That metronidazole can act rapidly was shown in two in-patients who were not included in the clinical trial. Daily examination showed that trichomonads had disappeared in 24 hours in one patient and within 48 hours in the other.

There is no evidence to suggest that any resistance developed to metronidazole. Four strains isolated

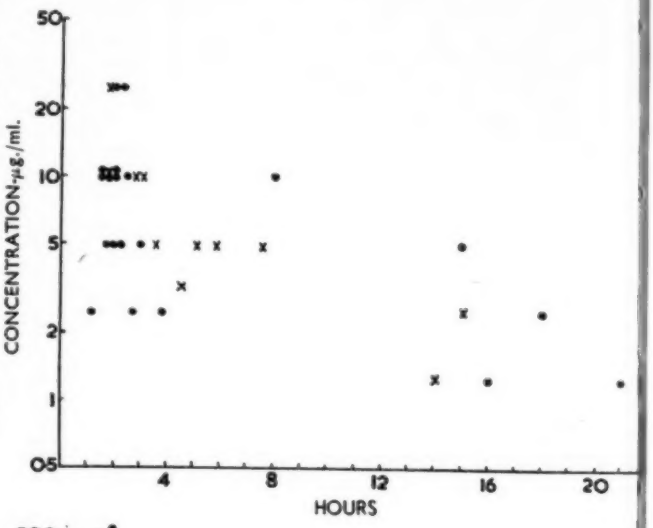

FIG. 4

FIG. 4. *Trichomonacidal activity of serum in 31 patients after completion of a course of treatment with metronidazole. (X indicates patients who did not respond to treatment.)*

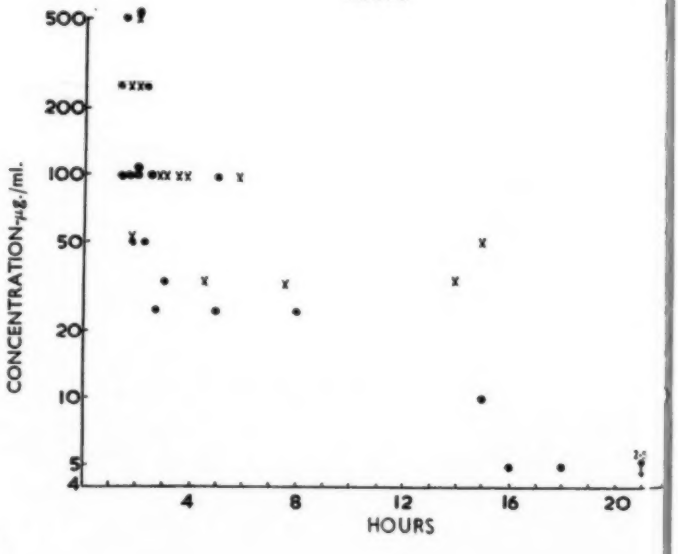

FIG. 5

FIG. 5. *Trichomonacidal activity of urine in 33 patients after completion of a course of treatment with metronidazole. (X indicates patients who did not respond to treatment.)*

after treatment from patients who had not responded had exactly the same sensitivity as the strain isolated before treatment.

It is also of interest to note that the sensitivity of the stock strain did not vary after 40 subcultures in medium without the drug being added.

Approximately 20% of patients treated with metronidazole reported minor side-effects (Watt and Jennison, 1960a), but examination of the cellular elements of the blood, liver function tests, and urine analysis revealed no abnormality. These investigations have not demonstrated any toxic effect due to metronidazole but final assessment must await more extensive use of the drug.

We wish to thank the consultant gynaecological staff of Saint Mary's Hospitals for their cooperation, and Dr. R. Forgan of May and Baker Ltd., who arranged a generous supply of metronidazole (Flagyl).

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Paper tests for occult blood in faeces and some observations on the fate of swallowed red cells

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SYNOPSIS Paper tests for occult blood were assessed on faecal specimens from adults. An orthotolidine/sodium perborate and a modified orthotolidine/peroxide test were found to be reasonably satisfactory. The Hematest and Occultest tablets and a paper guaiac test were unsatisfactory.

A large number of false positive results was obtained in children and infants. It is suggested that this might be due to meat derivatives reaching the faeces more easily in the young.

Red cells labelled with either Cr^{51} or Fe^{59} were swallowed by human volunteers. Although between 45% and 73% of the red cell iron reappeared in the faeces, the peroxidase activity of the labelled material dropped six-fold on passage through the alimentary canal.

The multiplicity of techniques for detecting occult blood in faeces suggests that none is entirely satisfactory, but the hitherto widespread use of the benzidine tube test implies that that is the method which best meets practical requirements. However, the manufacture of benzidine has been associated with the development of cancer in industrial workers (Baker, 1953) and as a result benzidine has recently become difficult to obtain. Of the possible alternative tests, the orthotolidine and guaiac paper techniques are simple and inexpensive, with the result that they are being increasingly used in wards and general practice, and in the laboratory the use of such methods eliminates much of the distasteful work, especially the cleaning of glassware. We have, therefore, carried out a series of tests designed to assess the reliability of some of the paper techniques and have compared them with tube benzidine tests. The sensitivity measurements have been based on the amount of altered orally administered blood in the stools as determined by the use of Cr^{51} labelled red cells rather than on artificial mixtures of fresh blood and faeces.

METHODS

TUBE TESTS FOR OCCULT BLOOD The benzidine tube test was performed on boiled faecal suspensions using varying dilutions of benzidine as described by Varley (1958).

PAPER TESTS FOR OCCULT BLOOD We performed four paper tests for comparison.

Hematest and Occultest Both were performed and interpreted according to the manufacturers'[1] instructions for the Hematest tablet.

Paper orthotolidine and sodium perborate test With the 'perborate test' of Abbott (personal communication) a very thin smear of faeces the size of a postage stamp was made in the centre of a Whatman No. 1 filter paper. About six large drops of a freshly prepared mixture of equal volumes of 2% sodium perborate in water and 2% orthotolidine in glacial acetic acid were added to the centre of the smear. A blue colour forming within two minutes outside the area of the faecal smear was regarded as positive. We found that the stock solutions could be kept at room temperature for about a week, after which the sodium perborate began to deteriorate.

Paper orthotolidine test This was performed as described by Moss (1955). Two modifications were made. First, the results were read after 30 seconds as many normal specimens gave a positive result after a longer time. Secondly, 2%, 1%, and 0·4% orthotolidine solutions were used as well as the 4% in an attempt to reduce the sensitivity of the test.

Paper guaiac test This was performed as described by Morgan and Roantree (1957).

NEGATIVE CONTROLS There were (1) normal adults on a normal diet excluding aspirin; (2) normal adults on a diet excluding meat and offal and taking no drugs. Collections were controlled by the use of carmine markers. Two of the volunteers in this group were shown to be free of significant gastrointestinal bleeding by estimating the radioactivity of their faeces after intravenous injection of Cr^{51}-labelled red cells. (3) Normal children on a normal diet; (4) normal children on a meat- and offal-free diet.

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[1] Ames Company Limited.

These had previously given positive benzidine tube tests on a normal diet. (5) Normal bottle-fed babies aged from a ½ to 5 months. This group was chosen to reduce errors due to bleeding maternal nipples, haemorrhagic disease of the newborn, and teething. The results from babies over 3 months of age have been placed in a separate category, since mixed feeding usually began at about this time.

POSITIVE CONTROLS Two normal adults who were on a meat- and offal-free diet and taking no drugs, swallowed known amounts of their own red cells, which had been labelled with a radioactive isotope Cr^{51} (Veall and Vetter, 1958). The red cells, suspended in milk, were drunk over a 12-hour period in order to simulate high gastrointestinal bleeding. The faeces were collected, weighed, and their radioactivity measured in a ring of six Geiger-Muller counters. This was compared with the radioactivity of a sample of the swallowed blood. The data so obtained were used to derive a figure for the concentration of blood giving rise to red cell residues in the faecal specimens.

In order to measure the absorption of iron after swallowing blood, red cells labelled with Fe^{59} were drunk, and the experiment continued as with the Cr^{51}-labelled blood. The red cells were labelled *in vivo* by the intravenous injection of Fe^{59} into a rabbit and venesection 10 days later, by which time some of the Fe^{59} had been incorporated in the haem radicle. The resulting rabbit red cells were washed with normal saline and mixed with the subjects' own blood to give the required volume.

One child (a negro hydrocephalic) continued to give strongly positive occult blood tests while on a diet. The child was excluded from the series as there was a history of anaemia, treated with intramuscular iron, and gastrointestinal bleeding was suspected. One baby (aged 2 weeks) was excluded from Table III because the health visitor noticed that it was passing black stools. The tests for occult blood were strongly positive.

RESULTS

NEGATIVE CONTROLS Tables I, II, and III show the results obtained.

POSITIVE CONTROLS The results obtained on two subjects on a meat-free diet are shown in Table IV. These subjects had swallowed amounts of Cr^{51}-labelled blood varying between 5 and 20 ml. The

TABLE I

RESULTS ON NORMAL ADULTS

				18 Adults on Normal Diet		*Four Adults on Meat-free Diet*	
				Total Specimens	*No. Positive*	*Total Specimens*	*No. Positive*
Tube tests	Benzidine		10%	83	12 (14%)	23	1 (4%)
			5%	83	5 (6%)	23	0
			3%	83	0	23	0
			1%	83	0	23	0
Paper tests	Orthotolidine	Hematest		83	48 (58%)	23	8 (35%)
		Occultest		83	73 (88%)	23	15 (65%)
		Perborate		80	11 (14%)	23	0
		Method of Moss	4%	70	56 (80%)	18	6 (33%)
			2%	56	24 (43%)	15	4 (27%)
			1%	69	6 (9%)	15	0
			0·4%	70	0	9	0
		Guaiac		62	1 (2%)	23	0

TABLE II

RESULTS ON NORMAL CHILDREN

				117 Specimens from 86 Children on Normal Diet No. Positive	*18 Specimens from Three Children on Meat-free Diet No. Positive*
Tube tests	Benzidine		10%	49 (42%)	1 (5·5%)
			5%	36 (31%)	0
			3%	24 (21%)	0
			1%	3 (2·5%)	0
Paper tests	Orthotolidine	Hematest		94 (80%)	1 (5·5%)
		Occultest		115 (98%)	10 (55%)
		Perborate		46 (39%)	1 (5·5%)
		Method of Moss	4%	106 (91%)	8 (45%)
			2%	86 (74%)	2 (11%)
			1%	63 (54%)	0
			0·4%	25 (21%)	0
		Guaiac		3 (2·5%)	0

TABLE III

RESULTS ON NORMAL INFANTS

				32 Specimens from 32 Babies Aged ¼ to 3 Months No. Positive	28 Specimens from 28 Babies Aged 3 to 5 Months No. Positive
Tube tests	Benzidine		10%	4 (12%)	9 (32%)
			5%	2 (6%)	5 (18%)
			3%	0	2 (7%)
			1%	0	0
Paper tests	Orthotolidine	Hematest		27 (84%)	21 (75%)
		Occultest		31 (97%)	28 (100%)
		Perborate		4 (12%)	6 (21%)
		Method of Moss	4%	16 (50%)	17 (61%)
			2%	7 (22%)	6 (21%)
			1%	2 (6%)	4 (14%)
			0·4%	0	1 (4%)
		Guaiac		1 (3%)	0

concentrations of red cell residues were calculated from the Cr^{51} content of the faeces. It will be noted that below a critical concentration of red cell residues (about 3·2 ml. blood/100 g. faeces) the majority of the tests were negative. The main exceptions to this were those tests giving numerous false positive results with the negative controls, *i.e.*, Hematest, Occultest, 4% and 2% Moss orthotolidine tests. The minimum amount of swallowed blood which gave the apparently critical concentration required for detection by the occult blood tests used was 10 ml. In every case the Cr^{51} label was excreted over a period of four days after ingestion, the maximum concentration occurring on the second day.

DISCUSSION

THE FATE OF SWALLOWED BLOOD In our experiments it was necessary to swallow 10 ml. of blood before it could be detected in the faeces. This was in agreement with some other authors, *e.g.*, Needham and Simpson (1952) even though our results were not strictly comparable, since the blood was taken over a 12-hour period instead of in one dose.

Needham and Simpson found that when blood was emulsified directly with faeces, the Gregersen test could detect a concentration of 0·5 ml. blood in 100 g. of faeces. We obtained a similar result with the tests employed by us. It is therefore of interest

TABLE IV

RESULTS OF OCCULT BLOOD TESTING ON FAECES OBTAINED AFTER SWALLOWING Cr^{51}-LABELLED BLOOD

Concentration of Red Cell Residues Expressed as ml. Blood/100 g. Faeces	Tube Tests: Benzidine 10%	5%	3%	1%	Paper Tests: Orthotolidine Hematest	Occultest	Perborate	Method of Moss 4%	2%	1%	0·4%	Guaiac
0·02	−	−	−	−	−	+	−	+	−	−	−	−
0·02	−	−	−	−	−	+	−	+	−	−	−	−
0·3	−	−	−	−	−	+	−	+	+	−	−	−
0·3	−	−	−	−	−	+	−	−	−	−	−	−
0·5	−	−	−	−	+	+	−	−	−	−	−	−
0·7	−	−	−	−	+	+	−	+	−	−	−	−
0·8	−	−	−	−	−	+	−	+	−	−	−	−
0·9	−	−	−	−	+	+	−	−	−	−	−	−
1·2	−	−	−	−	+	+	−	+	−	−	−	−
1·3	−	−	−	−	−	+	−	+	−	−	−	−
1·6	−	−	−	−	+	+	−	+	+	−	−	−
1·7	−	−	−	−	+	+	−	+	+	−	−	−
2·1	−	−	−	−	+	+	+	+	+	−	−	−
2·4	−	−	−	−	+	+	−	+	+	−	−	−
3·0	−	−	−	−	−	+	−	+	+	−	−	−
3·2	−	−	−	−	+	+	−	+	+	+	−	−
3·4	+	+	+	−	+	+	+	+	+	+	+	+
3·8	+	+	+	−	+	+	+	+	+	+	+	+
4·2	+	+	+	+	+	+	+	+	+	+	+	+
5·6	+	+	−	−	−	+	+	+	+	+	+	+
6·0	+	+	+	+	+	+	+	+	+	+	+	+
8·2	+	+	+	−	+	+	+	+	+	+	+	+

that when Cr^{51}-labelled blood was swallowed and the concentration of blood products in the faeces was assessed by the Cr^{51} label, the tests could detect only about 3 ml. blood per 100 g. faeces. This represents a six-fold drop in sensitivity.

If red cells are labelled with Cr^{51} the label becomes attached to the globin part of the haemoglobin (Gray and Sterling, 1950). When globin is digested in the alimentary tract, the Cr^{51} label would be set free and most of it is found in the faeces (Jones, 1958). In our experiments between 81% and 99% of the label was recovered. When the faecal blood concentration is calculated from the Cr^{51} content, the result will be too low because of the partial absorption of the label. The concentrations of red cell residues shown in Table IV are therefore a slight underestimate of the true figure.

The six-fold drop in peroxidase activity must be due to either or both of two factors, namely, (1) alteration of the haemoglobin molecule and (2) absorption of the iron-containing fraction.

In order to assess the part played by absorption of iron, the experiment was repeated using red cells labelled *in vivo* with Fe^{59}. During labelling, the Fe^{59} became incorporated in the haem and therefore the absorption of the label did represent absorption of iron present in the haemoglobin of the swallowed red cells. To avoid the radiation dosage associated with labelling in humans *in vivo*, rabbits' blood was used, but it is unlikely that this would invalidate the results.

In two experiments the recovery of the Fe^{59} label in the faeces was 45% and 73% after swallowing 10 and 20 ml. blood respectively. The marked drop in peroxidase activity in the digestive tract can, therefore, not be explained purely by absorption of iron. The authors found that neither digestion *in vitro* of blood with proteolytic enzymes (pepsin and trypsin) nor incubation in a faecal suspension resulted in any detectable drop in peroxidase activity. It is of interest that Bramkamp (1929) did show that digestion *in vitro* of blood with pancreatin reduced the strength of the colour reaction between haemoglobin and benzidine by about 50%.

In the present experiments the quantities of swallowed blood never exceeded 20 ml. With a large haemorrhage it is likely that neither digestion nor absorption would be so complete. This might account for the recovery of between 33% and 85% of the peroxidase activity by Andrews and Oliver-González (1942) after swallowing between 61 and 65 ml. of calves' blood. The site and agent responsible for the deterioration in the peroxidase activity of swallowed haemoglobin is still obscure.

RESULTS FROM CHILDREN Children not on special diets gave numerous false positives with all the tests (Table II). The results of Needham and Simpson (1952) on adults suggested that 'eating meat seldom accounted for a positive reaction' and 'it was necessary to feed patients with rather large amounts of meat rich in blood (liver and black pudding) before the faeces gave a positive reaction'. It is of interest that these children received no liver or black pudding and usually had one meat meal a day.

It may be that our results were due to immaturity of the digestive tract. Small amounts of meat would then produce faecal peroxidase activity. This is supported by the decrease which occurred when three children, chosen because of their previous positive results, were placed on a meat-free diet (Table II). There was also a tendency for the number of false positives to decrease with increasing age.

Positive occult blood tests on children on a normal diet appear to be of little diagnostic value.

RESULTS FROM NORMAL INFANTS The mothers of the babies in our series were advised to begin mixed feeding when the infants were about 3 months old. In order to attempt a rough division of the purely milk-fed babies from the babies on mixed feeds, Table III has been subdivided into those below and above this age. The benzidine tube, perborate, and 0·4% Moss orthotolidine tests showed some increase in the number of false positives when mixed feeding was begun. The presence of some positive occult blood reactions with the milk-fed babies might be explained by their having begun mixed feeding at an earlier age.

ASSESSMENT OF INDIVIDUAL PAPER TECHNIQUES Because of the numerous false positives obtained with children and babies the individual paper techniques were assessed on the results obtained on adults only (Tables I and IV). The small number of specimens obtained from normal adults on a meat-free diet was unavoidable.

The ideal test for occult blood would be sensitive, give a clear-cut result with no false positives, and be unaffected by diet. It was thought unlikely that any single test would fulfil all these criteria, and that two tests might be required, namely, an insensitive screening test unaffected by diet and a sensitive test requiring dietary preparation. However, Table IV suggests that all the tests which did not give frequent false positives with adults had approximately the same sensitivity. The exception to this was the 1% benzidine which appeared less sensitive. The value of the apparently sensitive tests, *i.e.*, Hematest, Occultest, 4% and 2% Moss orthotolidine tests is doubtful since they gave numerous false positives on normal adults even on a meat-free diet (Table I).

Hematest No mention of dietary restriction was given in the manufacturers' instructions. It was of

interest that 58% of specimens from normal adults on a normal diet gave a positive result. Even on the meat-free diet 35% of specimens were positive. The manufacturers did not give precise instructions about the interpretation of the results. The appearance of a blue colour on the paper, even if localized to the vicinity of the tablet, was interpreted as a positive. Any attempt to interpret only diffuse coloration as positive was difficult because of the numerous borderline results.

These results threw some doubt on the diagnostic value of this test.

Occultest This test is recommended by the manufacturers for detection of blood in urine. However, Cullis (1959) compared the Hematest, Occultest, and Gregersen paper benzidine test on faeces. He found that the Occultest read at 95 seconds gave an 88% agreement with the Gregersen test.

In our series 88% of specimens obtained from normal adults on a normal diet and 65% of specimens obtained from normal adults on a meat-free diet gave positive results when read at two minutes.

A random sample showed no discrepancy between results read at both 95 and 120 seconds. This test did not appear suitable for use with faeces.

Perborate test Fourteen per cent of specimens from normal adults on a normal diet gave a positive result. No positive results were obtained in dieted adults. Consistent positive results were obtained with concentrations of red cell residues above 3·4 ml. per 100 g. faeces. This appeared to be a satisfactory paper test.

Moss orthotolidine test The 4% and 2% orthotolidine solutions gave a high percentage of false positives. In contrast the 1% and 0·4% orthotolidine solutions gave few false positive results. Tests using these concentrations appeared to be satisfactory.

Guaiac test Although very few false positives were produced, the test was difficult to read and it therefore cannot be recommended.

We should like to acknowledge the advice given by Mr. N. Veall on work with isotopes. We should also like to thank Dr. T. V. Mendelsohn, the matron and nursing staff of Ladywell Nursery, and Miss L. Coombe, superintendent health visitor, Guy's Hospital, for kindly arranging for the collection of specimens, and Mrs. M. MacDonald for cleaning the large quantity of glassware used. We thank Dr. E. N. Allott, Dr. H. Lehmann, and Dr. W. H. H. Merivale for their interest throughout this investigation.

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Technical methods

Quantitative determination of A2 haemoglobin by paper electrophoresis using tris buffer

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Quantitative determination of A2 haemoglobin by paper electrophoresis using TRIS buffer is feasible. There is sufficient separation of haemoglobin A2 from haemoglobins A1 and A3 to allow quantitative estimation by this method. The technique is simple and sufficiently quantitative to be of clinical value.

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MATERIALS AND METHODS

Haemoglobin electrophoresis is performed between siliconized glass plates, on Whatman No. 3 mm. filter paper, using TRIS buffer at *p*H 9·2 (trishydroxymethylaminomethane 50·4 g., ethylenediaminetetraacetic acid 5·0 g., boric acid 3·8 g., distilled water 1,000 ml.) (Cradock-Watson, Fenton, and Lehmann, 1959), for four hours at 300 volts, 10 milliamperes. Haemoglobin is prepared as described by Chernoff (1955) and 0·02 ml. applied as a 2 cm. band on a single broad filter paper sheet. Several specimens may be run simultaneously. Upon completion of the run the paper is dried, the bands cut into strips and subjected to densitometric scanning (unstained) in a Spinco Analytrol scanner, model RB (B5 cam, slit width 1 mm.) (Figs. 1, 2, and 3). The amount of A2 haemoglobin is determined as the relative percentage of the total haemoglobin. A2 haemoglobin is selected as the area approximately half-way between A1 and A2.

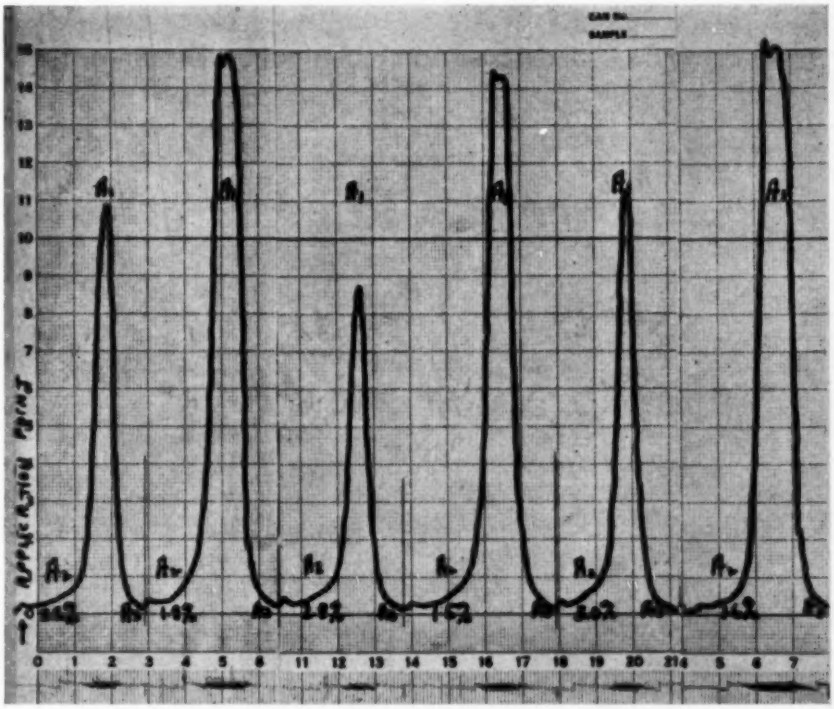

FIG. 1. *Illustrative examples of normal haemoglobin patterns after densitometric scanning.*

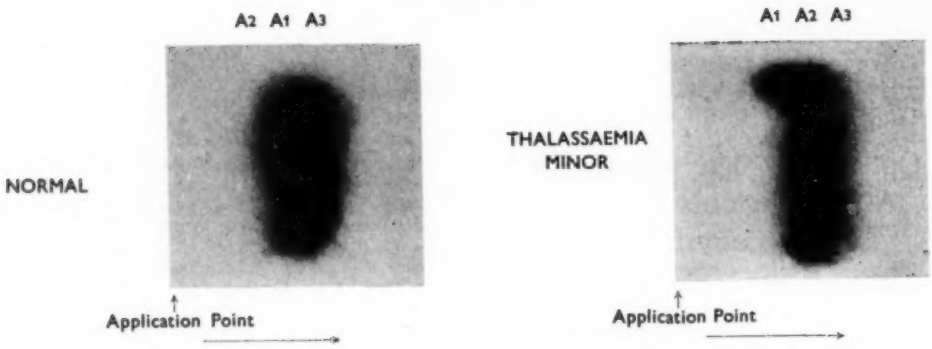

FIG. 2. *Haemoglobin pattern on paper electrophoresis in TRIS buffer, pH 9·2. Note prominent A2 haemoglobin in thalassaemia minor.*

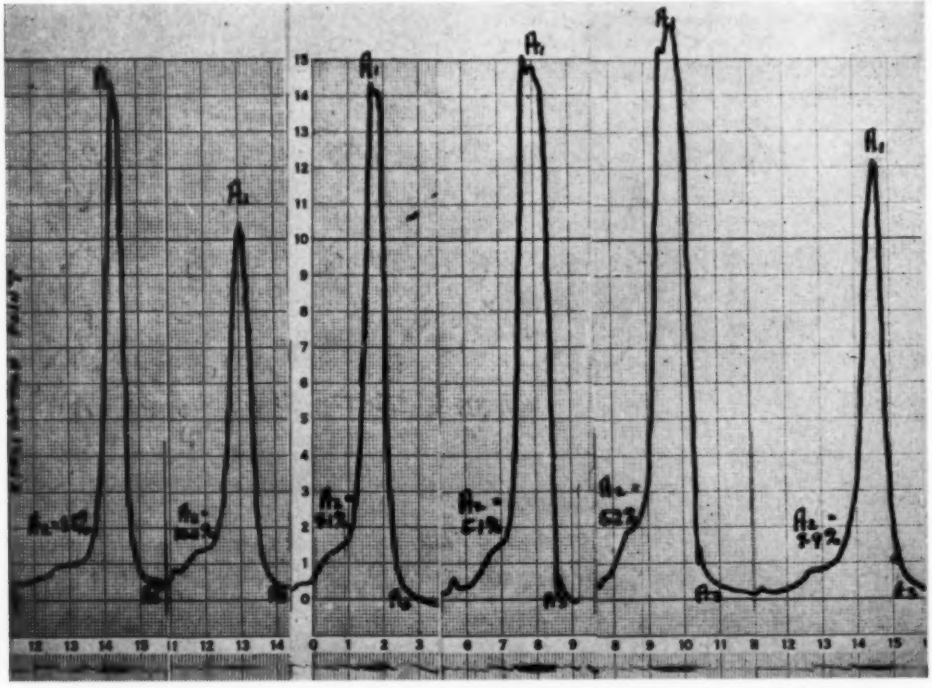

FIG. 3. *Illustrative examples of haemoglobin patterns in thalassaemia minor after densitometric scanning. Note prominent A2 haemoglobin.*

RESULTS

NORMALS In 52 normal adults the A2 haemoglobin ranged from 1·5 to 3·1%. The average value was 2·2% (Fig. 4).

THALASSAEMIA MINOR In 24 thalassaemia minor patients, the A2 haemoglobin ranged from 2·9 to 10·0% (Fig. 4). With one exception, in which a normal A2 value was found, the A2 haemoglobin level was raised in all

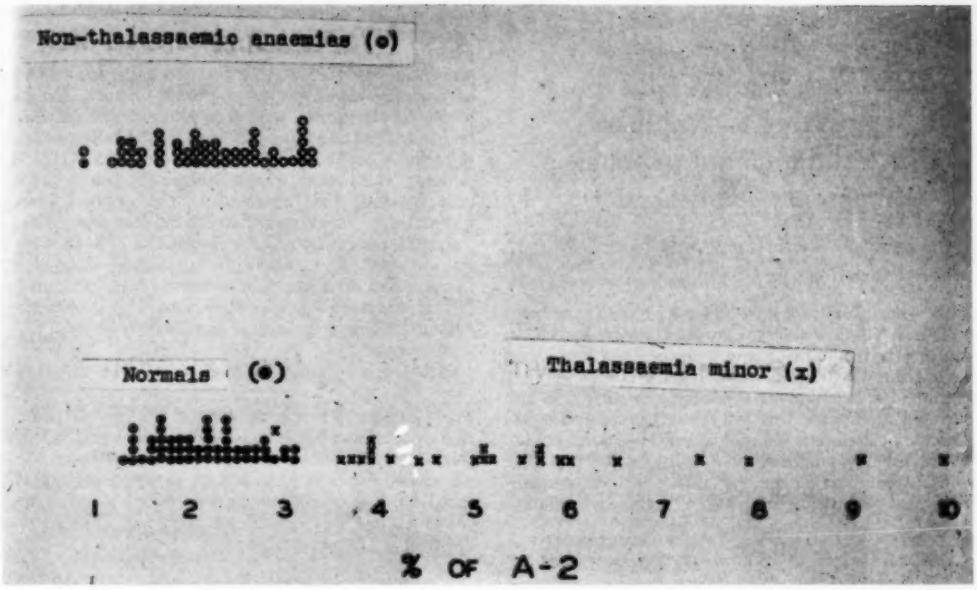

FIG. 4. *Values (%) in normal individuals, in patients with thalassaemia minor, and in patients with anaemias of non-thalassaemia aetiology.*

patients with thalassaemia minor. No thalassaemia major was encountered in this study.

NON-THALASSAEMIC ANAEMIAS In 52 patients with anaemias not due to thalassaemia, normal or subnormal A2 haemoglobin levels were found (Fig. 4). These anaemias were due to iron deficiency, infection, acquired haemolytic disease, uremia, marrow failure, abnormal haemoglobinopathies, etc. Low A2 levels were seen in two infants and in iron-deficiency anaemia. No patients with pernicious anaemia were seen in this study.

DISCUSSION

Quantitative determination of A2 haemoglobin by paper electrophoresis in TRIS buffer is feasible and simple. The values observed in most patients with thalassaemia minor are raised and sufficiently distinct from normal levels or those in other non-thalassaemic anaemias to merit its use in clinical practice.

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A method of rapid Rh typing employing the capillary tube technique and papainized incomplete anti-D sera

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The rapid capillary tube method for Rh typing introduced by Chown (1944) has the virtues of simplicity and of requiring the minimum of time, materials, and equipment, but has the disadvantages of requiring highly selected, potent and avid sera of the saline-agglutinating type. The following modification of the capillary tube technique, employing papainized incomplete anti-D sera, appears to have all the advantages of the original Chown technique without the disadvantage of having to use specially selected sera in order to reach a high standard of accuracy.

MATERIALS USED

ANTI-D SERA Two incomplete anti-D sera were used routinely in albumin as typing reagents. In addition 20 random incomplete anti-D sera, supplied by the Blood Group Reference Laboratory, all of which were found to be suitable as test reagents using the albumin replacement technique, were examined to determine the proportion which would be suitable for the modified capillary tube method.

PAPAIN SOLUTION Activated papain was used, made according to the method of Löw (1955).[1] For convenience, these details are repeated. Two grams of papain are ground in a mortar with 100 ml. M/15 phosphate buffer, *p*H 5·4. After filtration, 10 ml. 0·5 M cystein is added to activate the enzyme. The solution is then diluted with the buffer of 200 ml. and incubated at 37°C. for one hour. It is then ready for immediate use.

The papain solution was stored at −20°C. in small amounts sufficient for one day's use. Any unused at the end of the day was discarded and not re-frozen. This preparation has maintained its activity for more than a year and no significant deposit or turbidity has occurred.

CAPILLARY TUBES These were obtained commercially and have an internal bore of 0·5 to 1·0 mm. and a length of 8·0 cm.

METHOD

Papain solution, 0·03 ml., was added to 0·03 ml. of the incomplete anti-D serum under test, mixed in a precipitin tube and the mixture immediately run into capillary tubes in approximately 0·02 ml. amounts. Taking care to avoid air bubbles, an equal amount of a 20 to 25% saline suspension of once washed test red cells was run into each capillary with the serum-papain mixture. The ends of the capillary tubes were then plugged with plasticine, set in a rack at an angle of 45° with the red cell suspension uppermost, and placed in an incubator at 37°C. for 15 minutes. The pattern of the sedimenting column of red cells followed that obtained in the original Chown technique, *i.e.*, the D-positive cells showing a granular or 'beaded' appearance and the D-negative cells showing a smooth unbroken sediment. Incubation beyond 15 minutes had no advantage, although, depending on the test serum, positive results were noted in a shorter time, with a few sera in as short a time as five minutes.

EXPERIMENTAL TRIALS

The standard Rh typing method in this laboratory employs albumin-agglutinating anti-D sera, a tube technique, and an incubation time of two hours. This method was used in parallel with the modified capillary tube technique on 500 consecutive routine blood samples submitted to the laboratory for grouping. The results of this trial are shown in Table I.

TABLE I

RESULTS OF STANDARD RH TYPING METHOD

Tube Test Positive and Capillary Tube Test Positive	*Tube Test Negative and Capillary Tube Test Negative*	*Tube Test Negative and Capillary Tube Test Positive*	*Tube Test Positive and Capillary Tube Test Negative*	*Total*
420	79	1[1]	0	500

[1]The cells in this case were subsequently shown to be Rh type D[u].

Although during the period of trial the modified capillary tube method was used by experienced technicians, staff with limited experience in the use of serological methods and some junior staff with no previous experience of serology at all, it will be seen that the correlation between the tube technique and the modified capillary tube method is very good.

In order to discover the availability of incomplete anti-D sera suitable for this method, 20 random incomplete anti-D sera which gave satisfactory results using the albumin replacement technique were each tested against five D-positive and one D-negative blood sample. The results of this experiment are given in Table II.

From the results of this limited trial it appears that only one serum out of the 20 tested would be unsuitable for use by the modified capillary tube method. This suggests that a large number of sera which are suitable for use when diluted with albumin or in the albumin replacement technique are equally suitable for use in the modified capillary tube method with a great saving of time under emergency conditions. In addition to the 20 random sera tested in this series, one commercially produced anti-D

[1]In his original method Löw used Papayotin Merck 1:350, but the papain supplied by L. Light & Co., of Poyle Estate, Colnbrook, Bucks, was found to be perfectly satisfactory.

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TABLE II

RESULTS WITH ALBUMIN REPLACEMENT TECHNIQUE

Cells	1	2	3	4	5	6	7	8	9	10
O D positive	SP	SP	SP	SP	SP	SP	SP	D	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	D	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	D	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	—	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	D	SP	SP
O D negative	—	—	—	—	—	—	—	—	—	—

	11	12	13	14	15	16	17	18	19	20
O D positive	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP
O D positive	SP	SP	SP	SP	SP	SP	SP	SP	SP	SP
O D negative	—	—	—	—	—	—	—	—	—	—

SP = strongly positive D = doubtful

serum (Ortho slide-test anti-D) was tested and found to be satisfactory.

DISCUSSION

The principle of serum papainization has been applied by Hekker, Klomp-Magnee, Krijnen, and van Loghem (1957) to the development of a rapid slide technique for mass Rh typing. This method employs a papain solution rather different from that of Löw and uses larger volumes of serum than those needed for the capillary tube method. The authors report that over a series of 29,000 tests checked by the saline-albumin method no discrepancies were found using their papain-slide method. These workers also found the large majority of incomplete anti-Rh sera to be useful reagents. They reported that the papain solution employed tended to develop a white sediment and centrifuging was necessary before use. Apart from a faint opalescence which in no way interferes with the tests, no such difficulty has been encountered with the papain solution used in the present series of tests.

It has been reported by Löw (1955), and confirmed by Hekker and his colleagues (1957), that the serum-papain mixture loses its activity on storage and this has been my experience during these experiments. For optimum results the serum-papain mixture should be used immediately after mixing and certainly not later than three to four hours.

For a rapid Rh typing method to be satisfactory it must be capable of a high degree of accuracy, suitable for use with easily available typing sera, simple to perform, and easy to interpret. It should be free from technical difficulties even in relatively inexperienced hands and should give reliable results in the shortest possible time. While no method is likely to be absolutely foolproof the modified capillary tube method described here appears to satisfy these criteria, differing from the original technique of Chown in the use of the papainized incomplete anti-D serum. In addition it has the merit of using very small quantities of serum.

SUMMARY

A capillary tube method is described for rapid Rh typing based on Chown's technique and employing incomplete anti-D sera with the addition of activated papain.

When compared with a standard tube technique the method gave only one discordant result over a series of 500 random samples. This discrepancy was in respect of a blood subsequently shown to be of Rh type D^u.

Nineteen out of 20 incomplete anti-D sera tested proved suitable as typing reagents by this method.

The technique is simple and can be performed by relatively inexperienced staff, and very small quantities of serum are needed.

I would like to thank Miss K. E. Boorman and Miss B. E. Dodd for carrying out independent tests on the method, Miss Joan Lindars for her help in the experimental work, and Drs. M. Lubran and J. G. Selwyn for advice and criticism. Thanks are also due to Dr. Mourant, of the Blood Group Reference Laboratory, for supplying the unselected anti-D sera and to Ortho Pharmaceutical, Ltd., for supplying the commercial serum tested.

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The rapid slide agglutination test in the diagnosis of typhoid fevers and typhus[1]

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SYNOPSIS Of 10,000 sera examined for infectious diseases by both the rapid slide agglutination and the tube agglutination tests, 128 were found positive for typhus fever and 257 for typhoid and paratyphoid infections. Of 84 of the last mentioned cases of typhoid and paratyphoid, which were confirmed by cultural examination, 45 (53·5%) were negative to the tube test, whereas only one (1·2%) was negative to the slide test. Taking the total of 385 cases in which the clinical picture was typical and either one of the two serological tests positive, the tube test was negative in 183 cases (47·2%) whereas the slide test was negative once only (0·3%).

Of 201 cases in which both the slide and tube tests were positive, the slide test gave the result, on an average, six days earlier in 19 cases (10%).

A control test of 139 sera from patients suffering from various other diseases gave a negative result on the slide test.

Since the introduction of serological methods in the diagnosis of infectious diseases, it has become clear that the results of the serological tests do not always agree with the clinical evidence, and this is especially so in the case of the Widal serum tests in the diagnosis of typhoid fevers, and the Weil-Felix tests in the diagnosis of typhus fever.

The Widal serum test has several defects of which the following are the most important:—

Vaccination with TAB vaccine produces antibodies, usually of the 'H' type, in the vaccinated person, and the more efficient the vaccine, the greater the antibody response. In inoculated subjects cases have been recorded where positive Widal reactions were obtained in persons affected with a disease of a different aetiology, such as the common cold (Rappaport and Stark, 1954). Wilson and Miles (1946) pointed out that antibodies in the Widal reaction are detected in the blood of only 20% of the patients during the first week of the disease and up to 90% at the fourth week. This is, of course, a serious obstacle to rapid diagnosis and prevents early treatment. Moreover, it takes from 12 to 24 hours to complete a Widal test, which is a handicap in some cases where immediate treatment is indicated. There are instances on record in the history of typhoid and paratyphoid infection in which a positive Widal reaction did not appear at all. Werbin and Rappaport (1948) reported on an outbreak of paratyphoid A, in which the Widal test was consistently negative even with the antigen prepared from the Salmonella culture isolated from the blood of the patients.

The rapid slide agglutination test which is now used at the Hadassah Municipal Hospital, Tel-Aviv, as a regular method in testing sera from suspected typhoid, paratyphoid, and typhus patients, is believed to be an improvement on the Widal test in many respects.

MATERIALS AND METHODS

Ten thousand sera were examined during the period 1954-59. The hospital records of 385 patients suffering from typhoid, paratyphoid, or typhus fevers, were summarized. In every case cultures were made from blood, bone marrow, or faeces.

Each serum was tested against *Salmonella gallinarum*, *S. paratyphi* A, B, and C, and *Proteus OX* 19 and *OX* 2. We preferred to use *S. gallinarum* antigen for testing blood from suspected typhoid patients, because, having the same somatic antigen, it lacked the H antigen of *S. typhi* and was therefore unlikely to give false positive reactions in vaccinated persons.

REAGENTS The tetrazolium solution is a modification of Bendtsen's (1950) stained antigen for the detection of Brucella in milk by the ring test method and is prepared as follows.

Make a 0·2% solution of 2, 3, 5 triphenil tetrazolium in physiological saline and distribute it in sterile tubes in 8 ml. quantities. Sterilize in steam at 100°C. for 30 min., avoiding exposure to light during the process. Wrap in black paper and store at 4° C.

Glycerin phenol saline is made up as follows:—

Glycerin c. p.	1·0 ml.
Phenol, liquified	1·0 ml.
Saline 0·9%	98·0 ml.

Autoclave at 15 lb. pressure for 30 min.

Cultures were grown in 1,000 ml. Roux bottles on ordinary nutrient agar medium.

Proteus and *S. paratyphi* cultures were incubated at 37° C. for 24 hours and the *S. gallinarum* at room temperature for 48 hours. The growth in the bottles was then overlaid by 8 ml. of the tetrazolium solution, the bottles rocked gently to and fro to remove the growth from the surface of the agar, and incubated for a further 24 hours. The stained bacterial suspension was then distributed in centrifuge tubes, placed in a water bath at 60°C. for 30 min., and centrifuged for 15 to 30 min. to throw down the bacteria. The deposit from each Roux bottle was suspended in 10 ml. of glycerin-phenol solution and tested for sterility. The sterile suspension was tested against known positive and negative sera and stored at 4°C.

THE SLIDE TEST A drop of the patient's serum is placed on a clean glass slide opposite a drop of the particular

[1]This study is based on the work of the late Professor F. Rappaport, director of the Bacteriological Laboratory of Hadassah Hospital.

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TABLE I

RESULTS OF SLIDE AND TUBE TESTS COMPARED

	Total No. of Patients	*Slide Test Positive and Tube Test Positive*	*Slide Test Positive and Tube Test negative*	*Slide Test Negative and Tube Test Positive*
Typhoid and paratyphoid with positive cultures	84	38	45	1
Clinical evidence of typhoid and paratyphoid with negative cultures	173	82	91	—
Typhus fever	128	81	47	—
Total	385	201	183	1

stained antigen against which the serum is to be tested. The two drops are mixed with a glass rod, gently rocked to and fro for 30 to 40 seconds and read in an oblique light. Clumping of the bacteria in any of the antigens tested is an indication of the presence of antibodies specific to the particular antigen in the serum tested. Slight or late clumping is not considered to be of diagnostic value (Fig. 1).

The tube test was carried out in the ordinary manner using decimal dilutions of the patient's serum and a standard antigen for the agglutination test supplied by the Salmonella Centre of Jerusalem.

A titre of 1 : 100 or more was considered positive in the tube test.

RESULTS

More than 10,000 sera were tested, of which 257 were found positive for typhoid and paratyphoid and 128 for typhus fever. Table I compares the results between the rapid slide agglutination and tube tests in 385 cases.

It will be seen from Table I that out of 257 suspected cases of typhoid and paratyphoid, 84 were confirmed by positive cultures and of these only one (1·2%) was negative by the rapid slide agglutination test whereas by the tube test 45 sera (52·6%) gave a negative result. Of the 128 cases with a clinical picture of typhus fever, the slide test was positive in all of them whereas in the tube agglutination test only 47 (36·8%) gave a positive result.

To sum up, of the 385 cases proved by culture and those suspected on clinical grounds, the slide test was negative only once (0·3%) but the tube test was negative in 183 cases (47·0%). Of 201 cases in which both the slide and tube tests were positive, the slide test detected the presence of antibodies at an average of six days earlier in 19 instances (about 10%). In only one case did the tube test reveal the antibodies first. It is not, however, possible to draw definite conclusions from these comparisons, as the number of cases scrutinized is insufficient and because of the lack of exact data before the patient was admitted to hospital.

To check the specificity of the slide test, 139 sera were tested from patients suffering from other diseases and where no suspicion of typhoid, paratyphoid, or typhus fever existed. The slide tests of all the 139 sera were negative.

DISCUSSION

Welch and Stuart (1936), who were the first to use the rapid slide agglutination test for the diagnosis of typhoid and paratyphoid, tested 256 samples of sera from persons suffering from typhoid and an additional 200 sera from healthy persons who served as controls. They found excellent agreement between the slide and tube tests. They also found the slide test to be more specific and to give fewer doubtful results than the tube test in known negative cases.

Berger and Brecher (1939) tested 414 sera from patients suspected to be suffering from typhoid fever and found that all sera with a Widal titre of 1 : 80 or over gave a positive result on the rapid slide agglutination test. They further examined 100 sera from patients with an established diagnosis of typhoid fever, most of them during the second week of their illness or later, and found that in 98 cases the slide test was positive whereas the classical tube test gave a positive result in 68 cases only. Among these 100 sera were a few which were tested during the first week of illness when the slide test was already positive and the tube test was still negative. These results showed that whereas the slide test failed in 2% sera, the tube test missed 32% of them.

Rappaport and Stark (1955) examined more than 1,000 sera, of which 103 were positive to typhoid and paratyphoid and 123 to typhus fever. The results of the slide test were generally in agreement with the results of the tube tests when sera were taken from patients in or after the second week of illness. Sera taken before the second week of illness were usually positive by the slide test while the tube test was negative. Of 123 clinically diagnosed cases of typhus, 54 were positive in the slide test only and 69 were positive in both tests.

In our opinion the rapid slide agglutination test is superior to the tube test because it is specific. It is a more reliable test and gives a greater number of positive results in typical clinical cases and where the diagnosis was confirmed by cultural tests. Finally it is rapid (it takes a few minutes) and is easy to perform.

We wish to express our thanks to Mrs. Isa Gluckman, of the Department of Bacteriology and Serology, for her most valuable help in preparing the laboratory examinations.

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Haemoglobinometry by an automatic analytical procedure

M. G. NELSON AND A. LAMONT *From the Royal Victoria Hospital, Belfast*

In any busy laboratory providing a haematological service, the determination of haemoglobins by a manual method consumes much technical time. In our laboratory, out of a total of 79,000 routine haematological tests performed last year, 35% were haemoglobin estimations.

As part of a programme of automation of general haematology, we have, for six months, carried out the haemoglobin determinations with an Autoanalyser (Technicon Instruments Ltd.). This has proved easy, accurate, and time-saving. Our experience with this machine for this purpose has so impressed us that we are prompted to bring the procedure to the notice of others.

The method is fundamentally an automated colorimetric estimation of oxyhaemoglobin in solution. The sequence of events is shown in the flow diagram (Fig. 1).

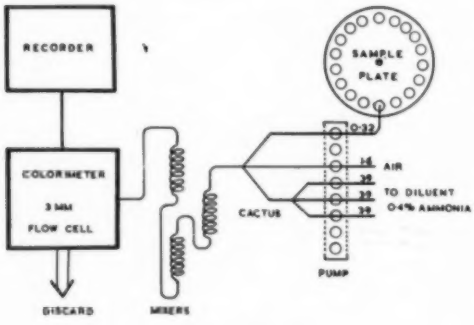

FIG. 1. *Flow diagram.*

Samples of blood are taken up by the sampling crook and after passing through the proportioning pump are mixed with 37 volumes of diluent. Complete mixing is achieved by a suitable arrangement of helical mixing coils. The diluted oxyhaemoglobin solution passes through a flow cell colorimeter and the imbalance in the latter is fed to an automatic graphical recorder.

REAGENTS

The only reagent required is 0·4% ammonia water. This concentration is used to achieve rapid haemolysis. It must be appreciated that all methods using the Autoanalyser require large volumes of reagents. Consequently, the diluent must be prepared in 10 litre amounts.

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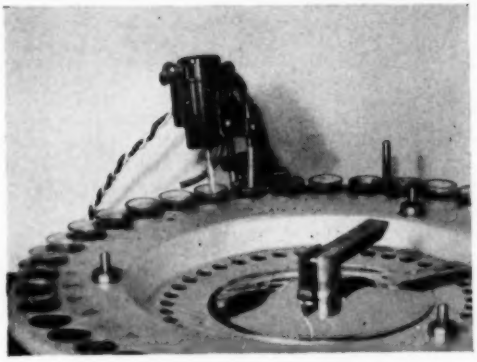

FIG. 2. *The sampler plate, crook, and mixer.*

SAMPLING

The samples of mixed venous blood collected in 5 ml. bijou bottles containing 10 mg. EDTA are mixed in a Matburn mixer. Approximately 1 ml. aliquots from each individual sample are poured into a clean perspex sample cup which is then placed on the sampler plate.

One difficulty which presented itself was the problem of the samples of blood settling in the cups before they were presented to the sampling crook. The method recommended by the American manufacturers of bubbling air through the samples led to streaming of bubbles without the blood being completely mixed. After consultation with the London agents they adapted the sampler crook to take a small motor with a Teflon paddle. This mixed the blood sample before it was presented to the sampling crook and proved both simple and satisfactory (Fig. 2).

The possibility that the motor paddle might carry over blood from one sample to the next was found not to occur since the rapid spinning of the non-wettable Teflon paddle made it self-cleansing.

THE MANIFOLD

The various components of the tube manifold and their sizes are shown in the flow diagram (Fig. 1). It will be seen that no dialyser or heating module are required. Instead three 'tumbler' coils are included in the circuit to ensure complete haemolysis. It was found that this arrangement of the manifold made it possible to run the sampler module at 40 tests per hour without alternating samples of water with samples of blood in order to achieve complete separation in the tube lines.

THE RECORDER

The peaks obtained on the electronic recorder should have clean apices and the curve should reach zero or thereabouts between each observation. Four blood

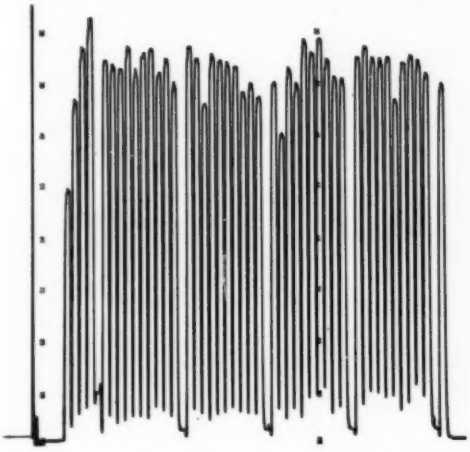

FIG. 3. *Graphical record of a run of tests, including the controls.*

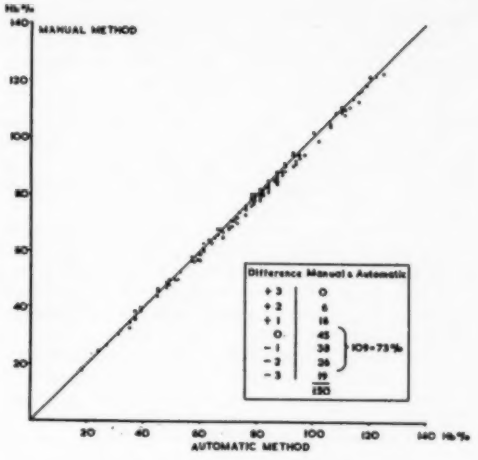

FIG. 4. *Comparison of results by manual and automatic methods.*

samples of known concentration are included in each run and from these results a standard curve can be plotted. This is used to determine the concentration of haemoglobin in the unknowns. To eliminate errors in recording we have found it convenient to divide a run into blocks of 10 samples separated by a water blank which acts as a marker (Fig. 3).

REPRODUCIBILITY

Preliminary experiments demonstrated the reproducibility of the machine. A specimen of blood when subsampled and tested by the machine gave identical peaks. The same series of samples of different concentrations of haemoglobin were tested on four successive days and the results for each sample agreed to ± 1%.

ACCURACY

The accuracy of the machine was tested by comparing the results obtained by the automatic procedure with a standard oxyhaemoglobin manual method in which the results were estimated with a standard photoelectric colorimeter. First the haemoglobin standard (Keeler) was estimated by both techniques and gave identical results. Then the haemoglobin concentrations of 150 individual samples of blood were determined in parallel by both methods. The results are plotted on a graph (Fig. 4) which shows excellent comparisons and linearity throughout the range of haemoglobin values. It can be seen that the differences between the results do not vary beyond ± 3% Haldane. On the whole, the automatic technique tends to give a haemoglobin value which, on average, is 0·15 g. lower than the manual method. This difference is not significant as the M.C.H.C.s calculated from both figures and the P.C.V. gave results in the same range.

DISCUSSION

Once these performance characteristics had been ascertained the automated procedure was put into routine practice and has been in constant use for six months.

It soon became obvious that a considerable saving on technical time was being achieved. To test just how much this was, we proceeded to test 100 routine blood samples by the manual and the automatic methods. The total time required from the start to the recording of the results was much the same by both methods, namely, 1 hr. 54 min. for the manual and 2 hr. 2 min. for the automatic. However, in the manual method the technician was fully employed throughout the whole period of 1 hr. 54 min., whereas in the automatic method only 37 min. of his time was needed, namely, 30 minutes in attendance to load the sampler plate and seven minutes to read the graph and record the results. During the remaining 1 hr. 25 min. the machine continued to take up samples and

TABLE 1

TECHNICIANS' TIME FOR 100 TESTS

	Manual[1]		
	hr.	*min.*	*sec.*
Pipetting diluent	—	21	25
Pipetting blood samples	—	47	30
Reading and recording	—	36	15
Washing pipettes	—	8	20
Total	1	53	30
	Automatic		
Loading sample plates	—	30	0
Reading and recording results	—	7	0
Total	—	37	0

[1]Average of three technicians

record the results automatically. As a result of this saving of technicians' time it has been possible to deviate the technicians into performing more complex procedures where automation has not been possible.

We are aware that the capital cost of installing this equipment for this single purpose may deter some management committees. For our part we are satisfied that the money expended has been justified in the accuracy of the results obtained and the saving of technical time simultaneously achieved.

The fundamental basis of any automatic device is that it must be in constant use during the working day in order to get maximum value from it. Our work load is such that we are able to keep the Autoanalyser fairly fully occupied every day doing the haemoglobin determinations alone. In smaller laboratories which cannot employ the machine whole time for this purpose, it is possible, by using the remainder of the apparatus, to carry out other chemical estimations for which the machine is specifically designed.

Another criterion of successful automation of a laboratory procedure is that the machine must be easy to operate and be free from maintenance and service difficulties. We have found during the six-month period that this automated procedure has been in daily routine use that it has been trouble-free. The only routine replacements so far required have been the ink for the graphical recorder, Teflon tubing for the manifold, and servicing of the minimotor stirrer. The latter becomes noisy after constant use and brushes require replacement. Eventually, the small motor will wear out but this replacement is simple and cheap. We have found that a new motor is required after two to three months' constant use.

The main criticism of the replacement of a manual by an automatic method in a hospital laboratory is that training of the junior technicians is impaired. This might be true of some techniques but is not so valid when levelled at haemoglobinometry, because the hand method has still to be retained for the occasional venous sample on which the result may be required in an emergency, as well as for the determination of haemoglobin in 'capillary' blood. As a result, the technician has still to perform the manual method but instead of carrying out a large number of haemoglobin estimations by hand he does only a percentage. This has, in our department, worked out at somewhat less than 2,000 haemoglobins per year. This number of tests is probably as many as a technician is likely to be called upon to do in a small laboratory. There seems little point in the endless repetition of a familiar method to the point of boredom and consequent possible inaccuracy.

In the development of this automated procedure, we should like to express our thanks first to our hospital committee for a generous grant towards 'automation' in our laboratory. This is but one of many uses to which this money has been put. The London agents of the Autoanalyser have been most helpful in setting up the method and solving the mixing problem on the sampler plate. All our technicians have contributed both interest and toil to the project which they now regard as amply rewarded.

Improvement of the uric acid determination by the carbonate method for serum and urine

F. EICHHORN, S. ZELMANOWSKI, E. LEW, A. RUTENBERG, AND B. FANIAS *From the Rappaport Laboratories, Biochemical Section, Beilinson Hospital, Petah Tikva, Israel*

SYNOPSIS An improved colorimetric procedure for determining uric acid by the carbonate method in serum and urine is described, using a 20% sodium carbonate solution with urea. Reliable results are also obtained in high concentrations of uric acid.

The first method for determining uric acid colorimetrically was based on the reduction of phosphotungstic acid by uric acid in the presence of concentrated sodium carbonate (Folin and Denis, 1912-13). Later the carbonate was replaced by cyanide (Folin, 1930) to intensify the colour of the reaction mixture to be read visually. But the cyanide method has several drawbacks: a poisonous solution must be handled, the colour developed is not always reproducible, at concentrations of uric acid above 6 mg. % the intensity of the developed colour is too weak and thus results are too low. When such high concentrations are encountered, the test must be repeated with smaller amounts of serum or diluted Folin-Wu filtrate. We observed the loss of sensitivity in the cyanide method at high concentrations of uric acid while experimenting with uricase. With uricase even 12 to 14 mg. % of uric acid could be detected, while with cyanide this could not be done unless smaller amounts of serum were used.

We tried using strong alkaline coupled with weak acid, such as sodium silicate, sodium borate, and sodium glycerophosphates, as a possible replacement for cyanide. The best results, however, were not obtained with these reagents but with sodium carbonate, as in the original method (Folin and Denis, 1912-13). Carbonate was also introduced anew by Caraway (1955). In his method the phosphotungstic acid reagent is used at a dilution of 1 : 10 and ensures the final alkaline *p*H of 10 needed for satisfactory development of colour. Under these conditions, however, the sensitivity of the method is decreased. For higher concentrations of uric acid (above 5 mg. %) this amount of reagent does not suffice and the standard curve ceases to be linear. A larger amount of the acid reagent may be introduced and kept at *p*H 10 and 20% of sodium carbonate instead of 10% is used. Under these conditions the colour is intensified. However, at this concentration of carbonate a white precipitate appears and makes the photometric determination impossible.

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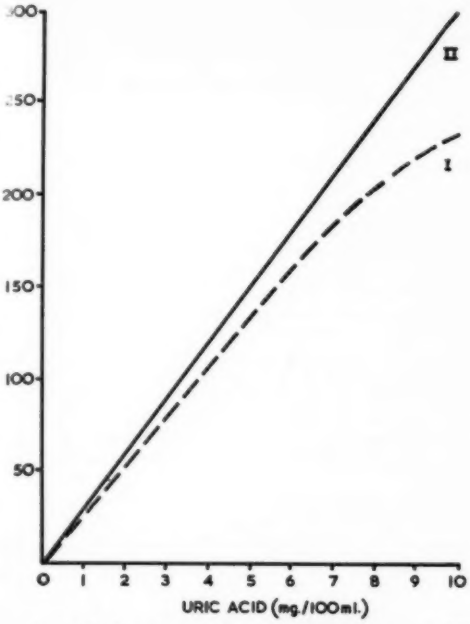

FIG. 1. *Colorimetric readings on Klett-Summerson 640 mμ:*
I With Caraway's reagents
II With the reagents of the present method

TABLE
COLORIMETRIC READINGS

Uric Acid (mg.%)	*With Caraway's Reagent*	*With Reagent of Present Method*
1	28	30
2	57	60
3	86	90
4	108	120
5	135	150
6	160	182
7	182	210
8	201	240
9	214	272
10	228	305

This difficulty has been surmounted by adding 15% urea to the sodium carbonate solution, as Brown (1945) did in the cyanide method. Thus the reagent may be added without precipitation while the *p*H stays at 10 and aids in the optimal development of colour.

In practice 0·1 ml. of the concentrated reagent produces the strongest colour with our urea-carbonate solution. The results are reliable and accurate in the highest concentrations of uric acid that occur in human blood.

REAGENTS

SODIUM TUNGSTATE Made up into a 2% solution in water.

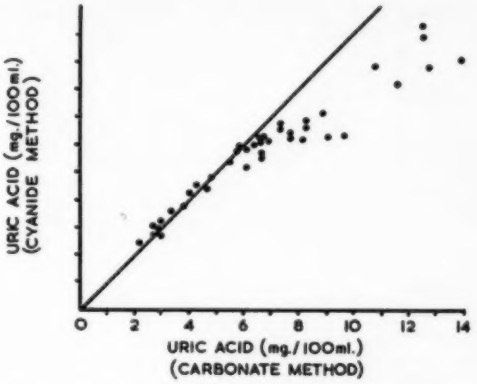

FIG. 2. *Comparison of the results obtained by the carbonate and cyanide methods applied to the same filtrates.*

2 ONE SIXTH N SULPHURIC ACID

3 SODIUM CARBONATE-UREA SOLUTION (*a*) Na_2CO_3, 30 g., is dissolved in water and made up to 100 ml. and (*b*) 45 g. urea dissolved in water and made up to 100 ml. Two parts of (*a*) are mixed with one part of (*b*), resulting in a 20% Na_2CO_3 to 15% urea solution. This mixture keeps well for two weeks.

4 PHOSPHOTUNGSTIC ACID REAGENT (FOLIN AND DENIS) One hundred grams of molybdate-free sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) are dissolved in 100 ml. of water, 80 ml. of 85% phosphoric acid is added, and the mixture is boiled gently with the aid of a reflux condenser for two hours. It is then cooled and diluted with water to one litre.

5 URIC ACID STANDARD For the stock standard (100 mg. per 100 ml.), 1 g. of pure uric acid and 0·6 g. of lithium carbonate are dissolved in about 150 ml. of water (warm if necessary). When completely dissolved 20 ml. of formalin 40% and 500 ml. of water are added. Finally, 25 ml. of 1/N sulphuric acid is added and made up with water to 1 litre.

WORKING STANDARDS Aliquots of the stock solution are diluted with water to provide standard solutions from 0·1 to 1 mg.%. For the standard curve 2 ml. of these solutions is treated exactly like the serum centrifugates and corresponds to 1 to 10 mg. % of uric acid respectively.

PROCEDURE FOR SERUM

Add to 0·3 ml. of serum 1·5 ml. of 2% sodium tungstate (1) and follow by 1·2 ml. of 1/6 N H_2SO_4 (2). Mix and centrifuge for 10 minutes. To 2 ml. of clear supernatant add 0·4 ml. of the carbonate-urea solution (3) and follow immediately by 0·1 ml. of the phosphotungstic acid reagent (4). For the blank, treat 2 ml. of water, and for the standard, each 2 ml. of the working standard solutions

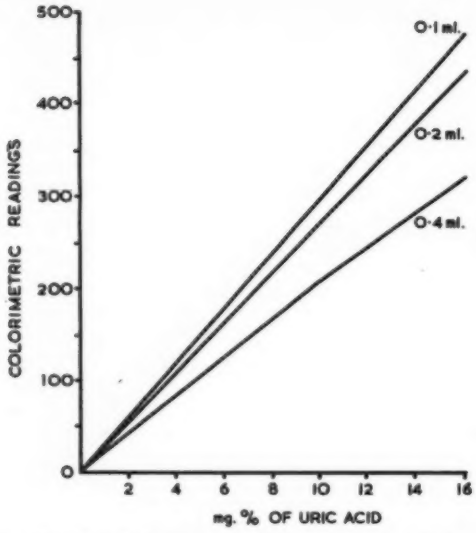

FIG. 3. *Standard curves with different amounts of phosphotungstic reagent calculated by the present method.*

(5) (corresponding to 5 and 10 mg. % uric acid) exactly as in the serum filtrate. Read after 10 minutes at 640 mμ in a photocolorimeter. We use a Klett-Summerson colorimeter coupled to a Kipp galvanometer (Rappaport and Eichhorn, 1955). Read the standard first as its colour slightly intensifies on standing. The samples should be read within the next 10 minutes.

The calculation is as follows:—

$$\frac{\text{Concentration of standard} \times \text{reading of test sample (unknown)}}{\text{Reading of standard}} = \text{mg. \% uric acid}$$

PROCEDURE FOR URINE

Add to 0·2 ml. of urine 12·3 ml. distilled water; follow with 0·5 ml. phosphotungstic reagent (4) and then with 2 ml. of the carbonate solution (3). For the blank, treat 0·2 ml. of water and for the standard 0·2 ml. of the half-concentrated standard (5) (corresponding to 50 mg. % uric acid) exactly as in the procedure for serum. Read and calculate in the same way as for serum.

Preliminary experiments in the development of this method were performed during the life time of the late Professor F. Rappaport, whose original guidance we gratefully acknowledge.

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Book reviews

MACKIE AND MCCARTNEY'S HANDBOOK OF BACTERIOLOGY. Edited by Robert Cruickshank. (Pp. xi + 980. 40*s*.) Edinburgh and London: E. & S. Livingstone. 1960.

'Mackie and McCartney' has enjoyed a well-deserved popularity for the past 35 years. There can be few departments of bacteriology where this handbook cannot be found on the shelves in a well-thumbed condition and it has played an important part in raising British medical bacteriology to the high esteem in which it is justly held throughout the world. To bring out a new and radically revised edition of such a work is no easy task, but one which Professor Cruickshank and his colleagues have achieved with brilliance. In spite of the use of smaller print in many places there are 303 more pages than in the 9th edition—an indication of the amount of new material included.

The intensely practical spirit of the book remains unchanged and the selection and description of the practical methods is excellent. This, combined with an up-to-date outlook and an almost equal competence in all branches, renders the volume without a peer in the technical bacteriological literature. In fact it is difficult to criticize it even in minor respects. In the brief section dealing with virulence the author could have emphasized that virulence can only be measured in relation to a species, and that such findings may be difficult to translate into terms of human disease. This concept is of particular importance in relation to mycobacteria.

On the purely technical side mention could have been made of the platinum tube dropping pipettes, as these are more accurate and more convenient than the all-glass ones. It is a pity that the semantic difficulty in the term 'Treponema complement fixation test' is continued. The Portnoy-type antigen obtained from *Treponema pallidum* is mentioned together with the antigen obtained from the non-virulent Reiter treponema, although Pillot and Borel have shown that at least one antigen specific to the Nichols strain exists. The section dealing with antibiotic interactions correctly defines synergism and antagonism as a greater or lesser bactericidal effect than equivalent concentrations of individual components alone, but unfortunately gives the method of Lamanna and Shapiro which reveals interaction at the bacteriostatic level only. However, Chabbert's method, which is the method of choice for antibiotic interactions, is also fully given. The chapters devoted to viruses, fungi, and protozoa are necessarily short. It is doubtful whether the inclusion of virus diseases in a text primarily devoted to bacteria can do justice to this vast and important subject. Within the necessary limitations of space, however, Dr. Swain has given a lucid and excellent summary of the more important human virus diseases.

Those of us who in one form or another are associated with the provision of a bacteriological service in the diagnostic or preventive fields are immensely grateful to

Professor Cruickshank and his colleagues for this excellent revision of an already superb text. To paraphrase a famous saying—seldom in the history of bacteriology has so much valuable information been provided for so modest a sum.

S. D. ELEK

ADVANCES IN CLINICAL CHEMISTRY, Vol. 2. Edited by Harry Sobotka and C. P. Stewart. (Pp. xiii + 387; 106 figures. $12.00.) New York: Academic Press. 1959.

This, the second volume in this serial publication, maintains and indeed raises the standard set by the first. Again there are assembled critical reviews of recent developments in seven specialized fields, each with a very full bibliography. Special mention of the comprehensive and authoritative section on paper electrophoresis is an expression of the reviewer's own interest rather than a comparison with the other sections. Indeed those dealing with blood ammonia, idiopathic hypercalcaemia, aminoaciduria, and bile pigments are valuable reviews, if less extensive, and the final section, on automation, deals informatively with a subject which looms ever larger on the horizon of the clinical chemist.

The book is well produced and will prove a valuable addition to the departmental bookshelves of hospital laboratories.

D. W. NEILL

CHEMICAL MICROMETHODS IN CLINICAL MEDICINE. By R. H. Wilkinson. (Pp. xiv + 121; 18 figures. 40*s*.) Oxford: Blackwell Scientific Publications. 1960.

This book describes the methods in use at the Hospital for Sick Children, Great Ormond Street, London. Most of them are methods in fairly general use, requiring 0·1 to 0·2 ml. of serum, and the majority are already described in other books. It might have been hoped than in a bench-book from this laboratory there would have been a full description of the ultra-micromethods which will be so valuable in studying chemical changes in newborn and premature infants, but this is confined to four pages giving the general principles of ultra micro-techniques but no more. The book is only 121 pages long and costs 40*s*., a price which will prevent its wide use.

E. N. ALLOTT

PATHOLOGY OF TUMOURS, 3rd ed. By R. A. Willis (xvi + 1,002 + index; 500 figures. 105*s*.) London: Butterworth. 1960.

The appearance of the third edition of Willis's *Pathology of Tumours* within 12 years of the first edition is itself evidence of the high esteem with which the work is generally regarded. There has been general revision of the text and some of the sections, including those on experimental carcinogenesis, carcino-sarcoma, bronchial carcinoma, chemodectomas, and endocrine tumours, have been rewritten or extended. No claim is made for a comprehensive bibliography but nevertheless 800 references have been added.

The value of this book to the clinician and to the candidate for higher examinations cannot be doubted. It is the more advanced pathologist seeking help in the more specialized fields of his subject who may occasionally be disappointed. The section on the gliomas for instance, which, as the author points out, is based on his own rather limited experience in this field, is not entirely satisfactory; and it is surprising to find that Kernohan's simplified classification, now so widely used in neurosurgical clinics, is not even referred to. Similarly the sections on tumours of bone, ovary, testis, and skin, though leaving the reader in no doubt of the author's views, do not always present a balanced account of the contemporary scene. Indeed this dogmatic presentation of personal opinions is at once both the strength and the weakness of the work.

T. CRAWFORD

QUANTITATIVE CELLULAR HAEMATOLOGY. By J. M. Yoffey. (Pp. xv + 122; 17 figures. 44*s*.) Oxford: Blackwell Scientific Publications; Springfield, Illinois: Charles C. Thomas. 1960.

The title of the monograph is strictly speaking inaccurate for it deals very largely with the interaction of the lymphoid tissues and the bone marrow. Professor Yoffey and his numerous co-workers have studied this problem for nearly 30 years at first in the dog and later in the guinea-pig. The quantitative methods described here were evolved to study this problem. Most haematologists are familiar with Professor Yoffey's thesis that the small lymphocyte, formed in the lymphoid tissue, enters the blood and thence passes to the bone marrow. This is clearly described. His next point, that the bone marrow small lymphocyte becomes a stem cell capable of forming red cells and granulocytes, is much more controversial. In particular, the striking lack of success in bone marrow restoration by thoracic duct lymphocytes in irradiated animals is much against this view. Despite all such objections, however, this is a fascinating work; the section on the role of the thymus in haemopoiesis, for example, being particularly interesting. One would like to point out, however, that not all small lymphocytes in macrophages have been phagocytosed; it has been abundantly shown that small lymphocytes may live inside such cells for long periods of time. This experimental observation is not even mentioned in this monograph.

J. G. HUMBLE

CORONARY VASODILATORS, Vol. 10. By R. Charlier. (Pp. xi + 208; 25 figures. 55*s*.) Oxford: Pergamon Press. 1961.

This monograph covers a field which does not strictly fall into the realm of pathology but is nevertheless of great importance to those interested in the functional aspects of coronary artery disease. It gives an up-to-date account of what is known of the physiological control of coronary artery blood flow and of the ways in which this can be modified by vasodilator drugs. The pharmacological properties of a great variety of substances which may affect coronary artery blood flow are described in some detail and there is a very full bibliography covering this branch of pharmacology.

A valuable feature which will be of interest to experimentalists is an account of the methods which may be

used either on the isolated heart or on anaesthetized intact animals for the measurement of coronary flow under various conditions.

T. CRAWFORD

Ciba Foundation Colloquia on Endocrinology, Vol. 13. HUMAN PITUITARY HORMONES. Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. xii + 336; 86 figures. 50*s*.) London: Churchill. 1960.

This is a report of the colloquium on human pituitary hormones held in Buenos Aires in August 1959 in honour of Professor B. A. Houssay. Nearly half comprises papers on human growth hormone and its metabolic effects. At present the significance of growth hormone in clinical medicine is far from clear, but the research which is going on may lead to results of clinical importance in the not too far distant future. The estimation of growth hormone has always been difficult, but an immunological method is described which may make it easier and lead to more rapid progress.

Other papers deal with pituitary gonadotrophins, A.C.T.H. and M.S.H., and thyrotrophic hormones.

Although much of this work has at present no direct bearing on clinical problems, it is very stimulating for the general reader to see what is going on in this important physiological field, and this report is an essential tool for all who are working on these problems.

E. N. ALLOTT

Broadsheets prepared by the Association of Clinical Pathologists

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. R. B. H. Tierney, Pathological Laboratory, Boutport Street, Barnstaple, N. Devon.** The prices include postage, but air mail will be charged extra.

1 The Disc Technique for the Rapid Determination of Bacterial Sensitivity to Antibiotics. 1952. R. W. FAIRBROTHER. 1s.

2 Determination of Sensitivity of *M. tuberculosis* to Streptomycin. 1952. R. L. VOLLUM. 1s.

3 The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents. 1953. L. C. NICKOLLS. 1s.

4 The Estimation of Carbon Monoxide in Blood. 1953. D. A. STANLEY. 1s.

5 The Identification of Reducing Substances in Urine by Partition Chromatography on Paper. 1953. G. B. MANNING. 1s.

6 The Paul-Bunnell Test. 1954. R. H. A. SWAIN. 1s.

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11 Mycological Techniques: (2) Cultural Isolation. 1956. R. W. RIDDELL. 1s.

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15 The Estimation of Faecal "Urobilinogen". 1957. C. H. GRAY. 1s.

16 Preservation of Pathological Museum Specimens. 1957. L. W. PROGER. 1s.

17 Cultural Diagnosis of Whooping-cough. 1957. B. W. LACEY. 1s.

18 The Rose-Waaler Test. 1957. C. L. GREENBURY. 1s.

19 The Laboratory Diagnosis of Fibrinogen Deficiency. 1958. R. M. HARDISTY. 1s.

20 Investigation of Porphyrin/Porphyria. 1958. C. RIMINGTON. 1s.

21 Quantitative Determination of Porphobilinogen and Porphyrins in Urine and Faeces. 1958. C. RIMINGTON. 1s.

22 Investigation of Haemolytic Anaemia. 1959. J. G. SELWYN. 1s.

23 The Dried Disc Technique for Bacterial Sensitivity Tests. 1959. R. W. FAIRBROTHER and J. C. SHERRIS. 1s.

24 Safe Handling of Radioactive Tissues in the Laboratory and Post-mortem Room. 1959. R. C. CURRAN. 1s.

26 The Periodic Acid-Schiff Reaction. 1959. A. G. E. PEARSE 1s.

27 The Laboratory Detection of Abnormal Haemoglobins. 1960. H. LEHMANN. 1s.

28 Daily Fatty Acid Excretion. 1960. A. C. FRAZER. 2s.

29 The Preparation of Bone for Diagnostic Histology. 1960. D. H. COLLINS. 2s.

31 Investigation of Haemorrhagic States with Special Reference to Defects of Coagulation of the Blood. 1961. E. K. BLACKBURN. 4s.

32 Detection of Resistance to Streptomycin, P.A.S., and Isoniazid in Tubercle Bacilli. 1961. R. CRUICKSHANK and S. M. STEWART. 2s.

34 Titration of Antistreptolysin O. 1961. H. GOODER and R. E. O. WILLIAMS. 2s.

J. clin. Path. (1961), **14**, 455

The artificial kidney and urea clearance

D. J. BLACKMORE AND W. J. ELDER

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SYNOPSIS A theoretical efficiency has been calculated from the known urea clearance of the artificial kidney, the assumed total body water of the patient, and the initial plasma urea.

The efficiency of dialysis never significantly exceeded the calculated figure. In some patients with trauma or glomerulonephritis the efficiency was very poor whereas in patients with renal failure complicating pregnancy the actual and calculated efficiencies were very close.

These observations are discussed and possible reasons for the failure of dialysis to bring down the plasma urea to the expected concentration are suggested.

We have been struck by the variable results of dialysis assessed in terms of plasma urea fall and clinical response while using a twin-coil artificial kidney during the past three years (Jackson, Bunker, Elder, and Joekes, 1960). It has been suggested that the variability of dialysis *in vivo* may be due to differences in blood flow rate and coil efficiency (Travenol leaflet provided with each disposable coil and Elliott, Horn, Kerr, and Pearson, 1961). Our experience indicated that these were unlikely to be a complete explanation, especially as post-obstetric patients consistently responded well to dialysis with high plasma urea clearances whereas some glomerulonephritic and post-traumatic patients had poor clearances and improvement in their clinical state was less satisfactory.

Haemodialysis has been extensively investigated *in vitro*, and parameters have been deduced which allow for mathematical analysis of artificial kidney function (Wolf, Remp, Kiley, and Currie, 1951). As sufficient data are available on most of our dialyses we have accordingly analysed them with respect to urea clearance.

MATERIAL AND METHODS

Haemodialyses were performed with a Kolff disposable twin-coil unit using a modified Travenol artificial kidney. The coil was sealed in the can with sponge rubber and the bath fluid circulating pump altered to give a constant flow of 34 litres per minute under variable load. The blood flow rate was measured by electromagnetic induction (Brennand and Bunker, 1960). Sodium and potassium concentrations in the dialysing fluid varied from patient to patient but no urea was added. Urea was estimated on heparinized plasma by a urease-nesslerization technique (King and Wootton, 1956). High plasma ureas (> 100 mg./100 ml.) were diluted before estimation. All tests were performed in duplicate with adequate control of recovery and standard solutions.

Dialyses which fulfilled the following criteria were selected for inclusion in this investigation:—

1, Clinically uneventful, uninterrupted six-hour dialyses; 2, dialysing fluid changed at two-hour intervals; 3, blood flow rate constant (±20 ml./min.) within the range 250 to 400 ml./min.; 4, circuit outflow pressure less than 80 mm. Hg; 5, patient's weight between 50 and 80 kg.; and 6, consistent biochemical data were available. Sixty out of 222 dialyses fulfilled these requirements.

If urea is evenly distributed and freely diffusible throughout the total body water it should be possible to calculate the expected plasma urea at the end of dialysis from the intitial plasma urea and the urea clearance of the artificial kidney. It would also be reasonable to assume that delay in intracellular fluid-extracellular fluid equilibration and the rate of urea production during dialysis would have little effect.

A nomogram (Fig. 1) was constructed to give the expected plasma urea at the end of dialysis assuming that the total body water was 57% of the body weight. The nomogram was based on the equation derived by Wolf *et al.* (1951) using standard nomographic technique. The following is an example of its use:

Let patient's pre-dialysis plasma urea = 400 mg.%
Let patient's body weight = 70 kg.
Let blood flow rate during dialysis = 300 ml./min.

then from nomogram

1 Setting 400 (initial urea Ao) on scale A and 70 kg. (body weight) on scale D the expected plasma urea at 2 hours is obtained at scale F against the 70 kg. line (240).

2 Repeat with Ao at 240, then at 4 hours expected plasma urea is 145.

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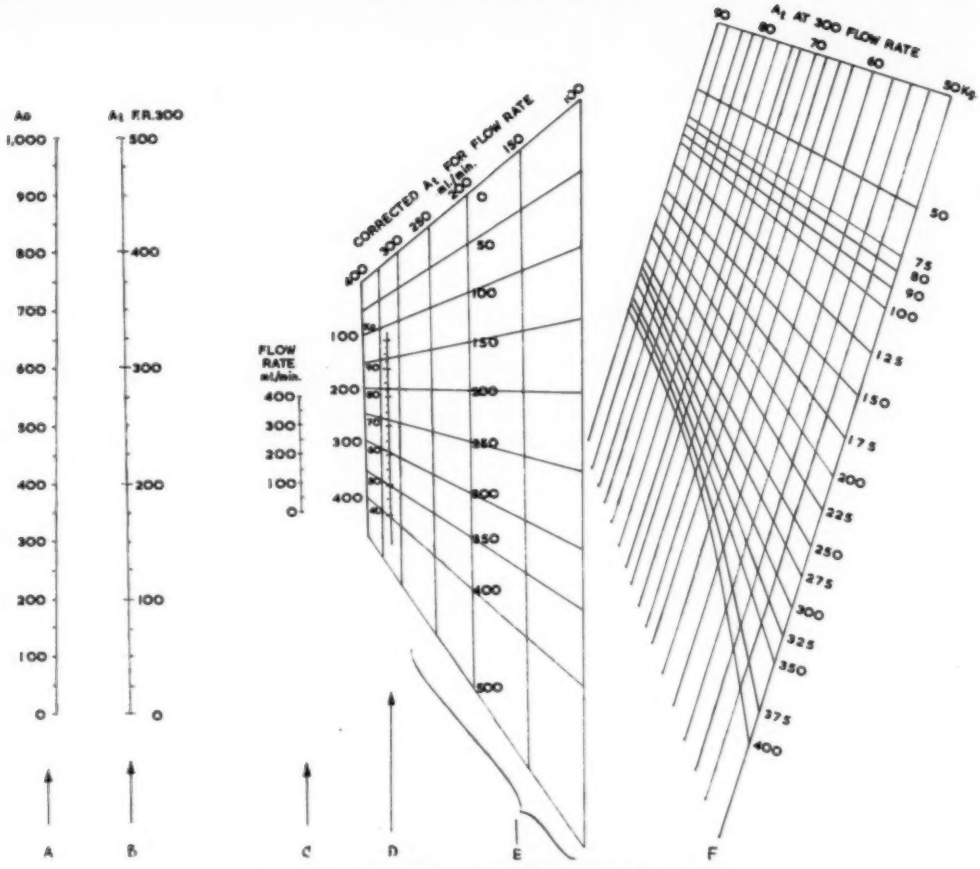

FIG. 1. *Nomogram for determining expected plasma urea.*

$$At = \frac{B}{B+V} \cdot A_o e - \left[\frac{B+V}{B} \cdot \frac{Ct}{V}\right] + A_o \frac{V}{B+V}$$

B = *dialysing fluid volume (ml.)*
V = *total body water 4/7 kg. (ml.)*
t = *2 hours (120 min.)*
C = *clearance (as found by Elliott* et al., *1961)*
Ao = *initial plasma urea (mg./100 ml.)*
At = *expected plasma urea (mg./100 ml.)*
e = *2·72*

3 Repeat setting Ao at 145, then at 6 hours expected plasma urea is 90.

Should the blood flow rate not be 300 ml./min., it is corrected at each stage utilizing scales B, C, and E in a similar manner.

So that comparison between dialyses would be possible a relationship between the calculated and observed post-dialysis plasma urea was derived as follows:

$$100\left[\frac{Uc - Uo}{Uc}\right] = \text{efficiency index}$$

Uc = calculated immediate post-dialysis plasma urea
Uo = observed immediate post-dialysis plasma urea

EXAMPLE Let the calculated immediate post-dialysis plasma urea (Uc) = 90 mg.%.

Let the observed immediate post-dialysis plasma urea (Uo) = 150 mg.%, then

$$\text{efficiency index} = 100\left[\frac{90 - 150}{90}\right] = -66$$

RESULTS

When the results of the 60 dialyses were assessed in this way it was evident that there was some relation

TABLE I

RELATION BETWEEN CLINICAL CONDITION, EFFICIENCY INDEX, AND RATES PLASMA UREA RISE

		Sex	*Age*	*No. of Dialysis*	*Day of Oliguria*	*Pre-dialysis Plasma Urea (mg.%)*	*Efficiency Index*	*Rate of Pre-dialysis Plasma Urea Rise*[1]	*Rate of Post-dialysis Plasma Urea Rise*[2]	*Ratio of Post- to Pre-dialysis Rates of Rise*
Traumatic										
G.H.H.	Multiple injuries	M	54	2	12	600	−55		0·85	
				3	18	500	−55		0·31	
				4	26	520	−16		0·33	
F.L.	Aortic valvotomy	M	58	1	9	350	−77	0·19	0·80	4·3
C.T.H.	Prostatectomy	M	61	1	7	380	−52	0·26	0·43	1·65
B.J.	Multiple injuries	M	21	1	7	350	−46	0·37	0·48	1·3
				2	14	390	−13		0·41	
J.W.K.	Multiple injuries	M	21	1	6	490	−60		0·48	
				2	9	550	−34		0·80	
A.V.	Resection of carcinoma colon	M	76	1	13	250	−14		0·21	
R.V.	Multiple injuries	M	36	1	6	365	−72	0·34	0·41	1·2
				3	14	560	−47	0·51	0·47	0·94
				4	18	500	−43	0·27	0·56	2·1
H.S.	Multiple injuries	F	16	1	9	370	−56	0·15	0·27	1·8
D.S.	Fusion cervical spine	F	43	1	13	370	−52		0·47	
				2	16	380	−69	0·29	0·44	1·5
A.D.	Facial injuries	M	19	1	5	400	−108	0·46	0·93	2·05
G.H.	Appendicectomy	M	64	1	6	390	−76		0·58	
				2	10	400	−36		0·55	
				3	15	540	−2	0·51	0·21	0·4
H.C.H.	Partial gastrectomy	M	63	1	6	335	−56		0·33	
				2	12	320	−62	0·16	0·44	2·7
				3	20	315	−22		0·14	
				4	36	420	−75		0·34	
C.H.S.	Partical gastrectomy	M	44	1	6	410	−93		0·81	
Glomerulonephritic										
R.S.	Chronic	M	22	1	14[3]	440	−61	0·25	0·29	1·15
P.L.	Subacute	M	52	1	11	430	−115	0·47	0·51	1·09
L.W.C.	Chronic	M	42	1		490	−42		0·49	
J.C.M.	Chronic	M	36	2	11	460	−140		0·55	
				3	16	470	−88		0·49	
				4	23	590	−92		0·21	
G.A.W.	Subacute	M	43	1	22	620	−69	0·82	0·38	0·45
				2	28	490	−63	0·376	0·41	1·1
				3	33	410	−133	0·15	0·57	3·76
J.L.	Acute	M	39	1	29	490	−40	0·31	0·28	0·9
R.N.	Chronic	M	62	2	12	500	−140	0·61	0·68	1·15
D.A.M.	Subacute	M	20	1	10	300	−94		0·32	
A.P.	Subacute	M	33	1	12	500	−40		0·14	
C.T.	Chronic	M	44	1	11	535	−110	0·83	0·59	0·71
Obstetric										
R.T.	Accidental haemorrhage 36/52	F	41	1	12	340	−14		0·20	
I.J.	Abortion 26/52	F	35	1	13	670	− 8		0·05	
				2	25	300	+ 3		0·15	
M.H.	Septic abortion 12/52	F	26	1	13	415	+ 3	0·39	0·27	0·69
S.S.	Accidental haemorrhage 36/52	F	23	1	11	355	− 6	0·24	0·25	1·05
				3	24	320	+15	0·21	0·34	1·6
J.V.	Eclampsia-stillbirth 28/52	F	22	3	26	410	− 7	0·31	0·26	0·84
Miscellaneous										
W.H.S.	Malignant hypertension	M	36	1	10	490	− 8	0·33	0·43	1·30
J.G.	Myocardial infarction	F	65	1	7	440	− 56	0·177	0·39	2·26
L.S.	Na ClO_3 poisoning	M	48	1	6	430	− 50	0·338	0·55	1·66
				2	11	490	−164	0·41	0·65	1·6
				3	18	540	− 31	0·242	0·29	1·2
				4	24	500	− 62	0·47	0·35	0·75
H.D.	Bacillary dysentery	M	51	1	9	560	−118	0·33	0·35	1·06
				2	14	520	− 27		0·30	
B.M.	M.T. malaria	M	36	2	9	460	− 22	0·42	0·61	1·45
				3	12	370	− 50	0·40	0·63	1·6
				4	16	500	−100	0·59	0·465	0·79
P.	C Cl_4 poisoning	M	56	1	9	450	−200	0·42	0·5	1·2
				2	16	465	−100	0·36	0·39	0·91
B.R..	Dissecting aneurysm	M	73	1	7	400	− 12	0·26	0·71	2·7

[1] Rate of plasma urea rise calculated for 48 hours pre-dialysis expressed in mg./kg. body weight/hour.
[2] Rate of post-dialysis plasma urea rise calculated for 40 hours post-dialysis, expressed in mgm/kg. body weight/hour.
[3] The onset of oliguria in the glomerulonephritic group was often insidious or not accurately known, thus the figures given are approximate.

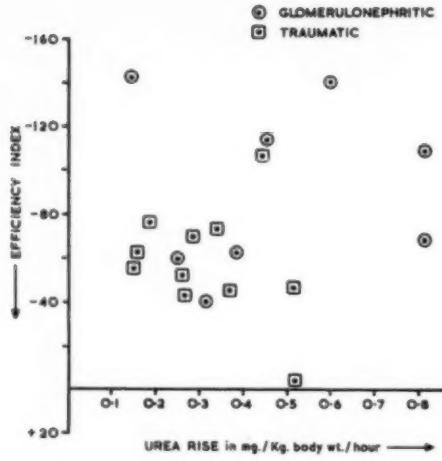

FIG. 2. *Efficiency index related to pre-dialysis rate of plasma urea rise for glomerulonephritic and traumatic groups.*

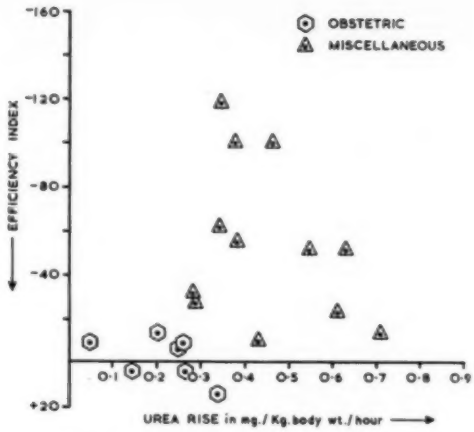

FIG. 3. *Efficiency index related to pre-dialysis rate of plasma urea rise for obstetric and miscellaneous groups.*

ship between the aetiology of the primary condition associated with the renal failure and the efficiency index of dialysis (Table I). The 25 dialyses on 13 patients with trauma had an efficiency index between -2 and -108, and, of these, 23 dialyses had an efficiency index between -2 and -80. The 14 dialyses on 10 patients with glomerulonephritis had an efficiency index between -40 and -140 and, of these, eight dialyses had an efficiency index between -80 and -140. The seven dialyses on five post-obstetric patients had an efficiency index between -14 and $+15$. The remaining 14 dialyses on seven patients with other conditions had a wide scatter of efficiency indices from -200 to -8.

EFFICIENCY INDEX RELATED TO PRE-DIALYSIS RATE OF PLASMA UREA RISE The efficiency index in the four groups was plotted against the pre-dialysis rate of plasma urea rise for the 48 hours before dialysis (Figs. 2 and 3).

No correlation was found and no significant difference was apparent between the groups.

EFFICIENCY INDEX RELATED TO POST-DIALYSIS RATE OF PLASMA UREA RISE The efficiency index was also plotted against the post-dialysis rate of plasma urea rise for the 40 hours after dialysis (Figs. 4 and 5). A significant correlation existed between the dialysis efficiency index and the post-dialysis rate of rise of urea in the traumatic and glomerulonephritic groups. There was also a statistically significant difference between these two groups ($t = 4{\cdot}08$ and $p < 0{\cdot}001$). The difference between the obstetric patients and the other two groups was obvious.

EFFICIENCY INDEX RELATED TO RATIO POST-DIALYSIS: PRE-DIALYSIS RATES OF PLASMA UREA RISE The ratio of the post-dialysis to the pre-dialysis rate of plasma urea rise was plotted against the efficiency index in the four groups (Figs. 6 and 7). Again there was no correlation in any group between the ratio and efficiency index.

Figure 6 shows that, ignoring the efficiency index, there was a difference between the traumatic and glomerulonephritic groups in respect of the post-dialysis to pre-dialysis rate of rise. It appears that traumatic patients had a significant increase in the rate of plasma urea rise after dialysis.

EFFICIENCY INDEX RELATED TO DAYS OF OLIGURIA In the traumatic and glomerulonephritic groups the dialysis efficiency index was plotted against the time in days after the onset of oliguria (Figs. 8 and 9). No obvious correlation appeared to exist between the efficiency index and days of oliguria in the glomerulonephritic or obstetric groups. In the trauma group the dialyses tended to become more efficient with increasing length of oliguria, with the exception of one patient (H.C.H., Table I) dialysed on the thirty-sixth day from onset of renal failure, who had begun a diuresis (>1 l. urine volume/24 hours) four days before. It is difficult to decide

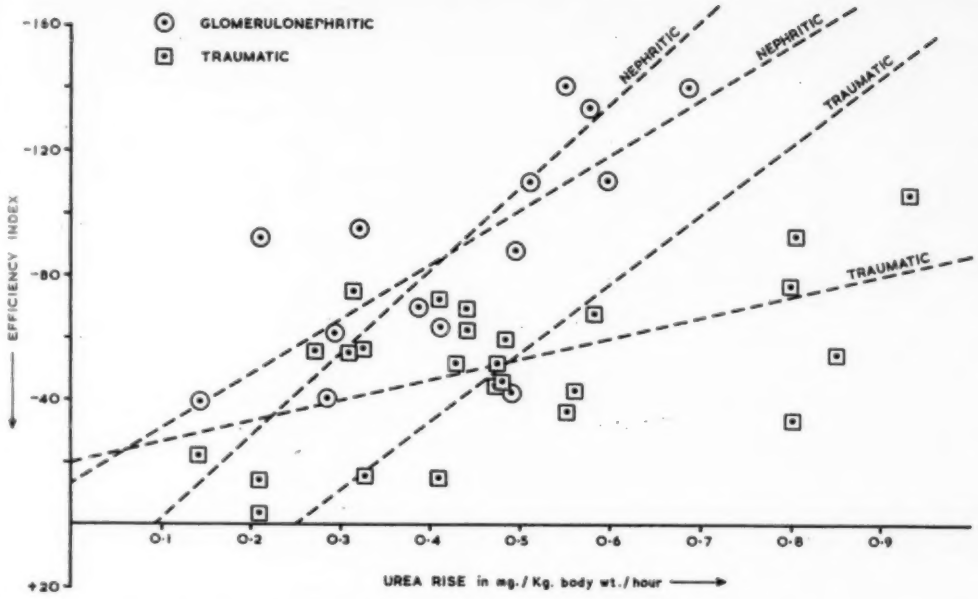

FIG. 4. *Efficiency index related to post-dialysis rate of plasma urea rise for glomerulonephritic and traumatic groups. For glomerulonephritic, r = 0·72, and for traumatic, r = 0·54.*

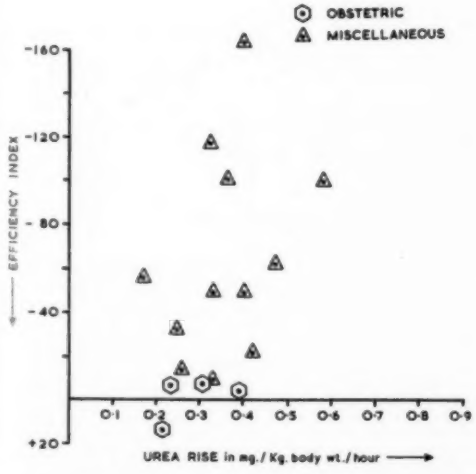

FIG. 5. *Efficiency index related to post-dialysis rate of plasma urea rise for obstetric and miscellaneous groups.*

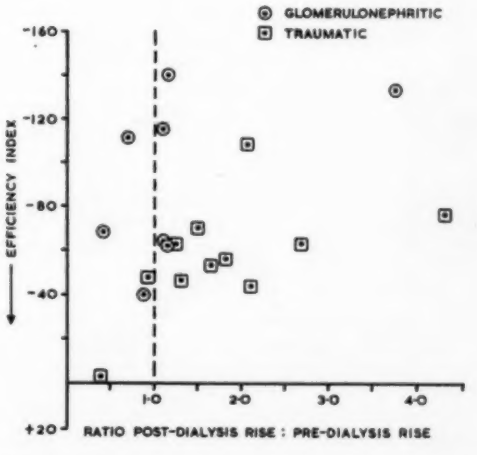

FIG. 6. *Efficiency index related to the ratio post-dialysis: pre-dialysis rate of plasma urea rise for glomerulonephritic and traumatic groups.*

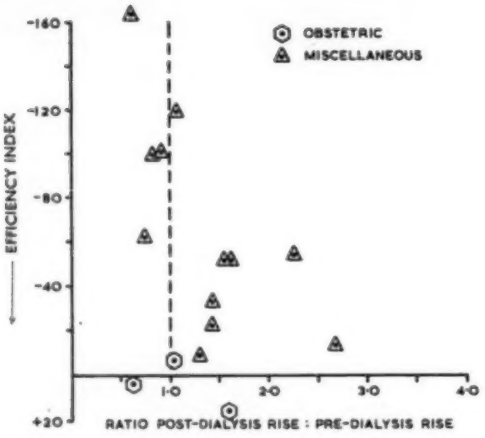

FIG. 7. *Efficiency index related to the ratio post-dialysis: pre-dialysis rate of plasma urea rise for obstetric and miscellaneous groups.*

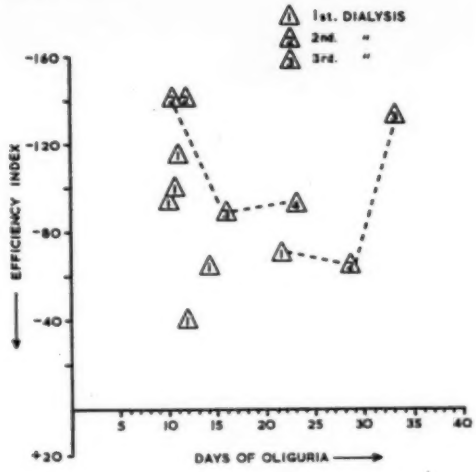

FIG. 9. *Efficiency index related to days of oliguria in the glomerulonephritic group. The dotted line indicates series dialyses on the same patient.*

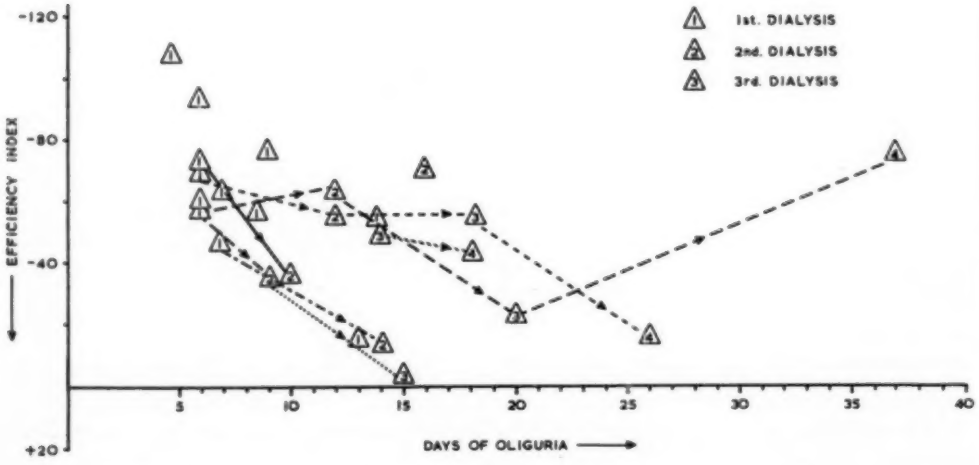

FIG. 8. *Efficiency index related to days of oliguria in the traumatic group. The dotted line indicates series dialyses on the same patient.*

TABLE II

PREDICTED AND OBSERVED UREA REMOVED AT DIALYSIS

		No. of Dialysis	*Theoretical Urea Removed (g.)*	*Actual Urea Removed (g.)*	*Actual:Theoretical Urea Removed (g.)*
Traumatic					
S.H.	Multiple injuries	1	110	101	0·92
		2	119	69	0·58
		3	87	109	1·25
C.T.H.[1]	Prostatectomy	1	60·5	88·6	1·46
J.N.	Multiple injuries	1	70·5	101·1	1·43
		2	68·5	65·1	0·95
B.J.[1]	Multiple injuries	1	104	112·2	1·08
		2	114	73·3	0·64
J.W.K.[1]	Multiple injuries	1	87	130	1·49
		2	140	135·7	0·97
Glomerulonephritic					
J.E.H.	Acute	1	86·2	46	0·53
W.B.	Chronic	1	92	80	0·87
		2	96·6	82	0·85
V.C.	Chronic	1	90·4	120	1·33
R.N.[1]	Chronic	1	110	135·6	1·23
W.W.	Acute	1	233	179·4	0·77
C.C.	Chronic	1	39·2	43·75	1·15
Obstetric					
I.J.[1]	Abortion 26/52	1	76	84·6	1·1
J.S.	Post-partum haemorrhage	1	93·2	81	0·86

The theoretical urea removal was calculated from the plasma urea fall observed across dialysis assuming that the total body water was 57% of the body weight.

[1]The dialyses of these patients are also included in Table I.

whether the improving plasma urea clearance was a function of time or whether it was related to previous dialyses.

PREDICTED AND OBSERVED UREA REMOVED BY DIALYSIS Unfortunately an accurate estimate of the total amount of urea removed by dialysis is only available in 19 dialyses (Table II), and only five of these patients could be included in the present paper. In general the results are equivocal, but in the traumatic group the urea removed during the second dialysis was less than that removed by the first dialysis.

DISCUSSION

It is of interest that only those patients with acute renal failure complicating pregnancy behave in the predicted manner with respect to urea clearance. Also it appears from only two estimations of urea removal by dialysis (Table II) that they lose their urea to the dialysing fluid in a similarly predictable way.

We have found that patients with acute renal failure complicating trauma have a poor plasma urea clearance on their first dialysis with the predicted or more than the predicted amount of urea removed and an improved clearance with a lower urea removal in subsequent dialyses. They also showed a significantly greater post-dialysis rate of plasma urea rise than the pre-dialysis rise and the less efficient their dialyses the greater was the post-dialysis rate of plasma urea rise.

The least efficient plasma urea clearance in this series was found in patients with glomerulonephritis. Those patients with renal failure of aetiology other than the three groups mentioned presented a wide range of results, the majority of which deviated from the predicted figures.

In trying to interpret these anomalous findings the postulate made by Merrill (Merrill quoted by Mather, 1960) that, in some patients, dialysis releases a metabolic block to the production of urea should be considered. If this is to account for the variation in plasma urea clearance enormous quantities of urea would have to be produced during dialysis to affect materially the calculated result. The rate of urea production in the body of a normal man weighing 70 kg. is of the order of 1 g. per hour; the production of 6 g. of urea in as many hours would only increase the blood urea by 15 mg./100 ml. A urea production as high as three times normal could not significantly affect the efficiency index. Also, it might be expected that the post-dialysis rate of plasma urea rise would be much greater than the pre-dialysis rate. Although this was seen in the traumatic group (see Fig. 6) it was

not found in most of the glomerulonephritic group where an efficiency index of between −80 and −140 was common. In the latter group it was also observed, with only one exception, that the calculated urea removal by dialysis approximated the actual removal.

Another possible explanation is that urea exists both in cell and plasma in diffusible and non-diffusible forms and that there is a greater ratio of non-diffusible to diffusible urea in the cell than in the plasma, these levels being maintained in equilibrium. The ratio might be determined by the aetiology of the primary condition.

Therefore on dialysis the following observations would be made: 1 An increased ratio of non-diffusible to diffusible plasma or cell urea would lower the efficiency index. 2 In the traumatic group an increased ratio of non-diffusible to diffusible cell urea before dialysis might result in a higher cell than plasma total urea concentration. If this is so the total body urea concentration would be higher than that calculated from the plasma urea level. Therefore more urea could pass into the dialysing fluid than predicted from the plasma urea drop. Thus it is not necessary to postulate the release of a metabolic block to the production of urea to account for the excess urea in the bath. During dialysis the equilibrium would be disturbed and a subsequent dialysis following re-establishment of an equilibrium at a lower non-diffusible level would result in a better efficiency index with a lower recovery of urea from the bath than was found formerly. 3 In the glomerulonephritic group a greater ratio of non-diffusible to diffusible plasma urea but a lower ratio of non-diffusible to diffusible cell urea than in the traumatic group might result in a lower efficiency index, with an approximate theoretical urea recovery from the bath and a lower post-dialysis rise in plasma urea.

CONCLUSION

We conclude from our results that it is difficult to accept urea as a freely diffusible, rapidly equilibrating substance in all patients. Only in the obstetric group does this assumption appear to allow accurate prediction of dialysis results.

The results from our other groups suggest that in some circumstances this is not wholly true and that urea might exist in both diffusible and non-diffusible forms. We already have some experimental evidence supporting this thesis but so far too few cases to justify any firm conclusion but we hope that publication at this stage might interest other units working in this field.

Following the preparation of this paper for publication Murdaugh and Doyle (1961) have reported the binding of urea by haemoglobin and albumin. They also showed that the quantity of urea bound was related to the urea concentration, indicating that the urea-binding ratios of haemoglobin and albumin will not become saturated in the range 10 to 400 mg. urea/100 ml. These authors also report that others have suggested that protein will bind urea. Ralls (1943) showed that urea was not equally distributed between red cell water and plasma water and found an average distribution of 1·14 : 1·00. Pasynskii and Chernyak (1950) reported that the adsorption of urea on albumin and globulin was not related to their respective water content and suggested that on a molar basis one urea unit will bind with two amino-acid residues.

We feel therefore that it is possible that protein binding of urea could be an explanation of its apparent non-diffusibility. It is also possible that a factor controlling the degree of binding may vary with the aetiology of the primary condition preceding renal failure.

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A new principle applied to the determination of calcium in biological materials by flame photometry

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SYNOPSIS The effect of magnesium sulphate in releasing calcium emission from interference by phosphate and sulphate has been investigated.

Samples were diluted in 10 mM $MgSO_4$, 2 mM NaCl, giving final calcium concentrations of about 0·05 to 0·10 mM. In this diluent, galvanometer readings were proportional to calcium concentrations up to 0·4 mM. The magnesium sulphate released calcium emission from depression by phosphate and sulphate. The excess sodium chloride eliminated enhancement of calcium emission by added sodium and potassium in the sample. Subtraction of background readings excluded direct interference.

A 3% correction was made for the effect of the viscosity of 1 : 50 plasma dilutions. Satisfactory recoveries of added calcium were obtained from plasma, urine, and faeces using the diluent described above. Results on urine and faeces correlated closely with those obtained by an EDTA titration method. Results on plasma were consistently 2% higher by flame photometry than by EDTA titration.

Other methods of calcium determination, depending on the use of radiation buffers or standard addition, were found to be unsatisfactory because of variable interference by phosphate at different calcium levels.

The measurement of calcium in biological materials by flame photometry is more difficult than that of sodium and potassium because of its lower emission energy and more serious interference by contaminating substances. The first difficulty can be overcome by using a high flame temperature, a prism monochromator, and a sensitive detector. Instruments incorporating these features permit the use of the weak calcium line at 422·7 mμ where interference is less than using the calcium oxide bands at 554 mμ and 622 mμ. Even at 422·7 mμ, however, there are three important sources of interference as follows:—

(1) Background (direct) interference: using a prism monochromator, this is due mainly to heterochromatic spectral radiation by sodium and potassium, only a fraction being due to scattered light.

(2) Enhancement or depression of calcium emission (indirect interference): the emission by calcium itself is enhanced by sodium and potassium and depressed by certain anions, notably phosphate and sulphate.

(3) Alteration of the physical properties of the solution because the quantity and dispersal of the spray reaching the flame may be influenced by differences in viscosity and surface tension due to protein and other organic compounds.

Many procedures for measuring calcium by flame photometry involve prior separation of calcium from interfering substances by precipitation of calcium oxalate (Powell, 1953; Chen and Toribara, 1953; Butterworth, 1957; Woollen and Walker, 1959) or by means of a cation exchange resin (Brabson and Wilhide, 1954; Denson, 1954; Jackson and Irwin, 1957). These preliminary steps are time-consuming and have errors of their own, thus combining disadvantages of other methods for calcium determination with those of flame photometry, while losing the simplicity of the latter.

Apart from the separation of calcium from interfering substances, many methods have been introduced to control their effects, but none has been generally accepted. The complexity of interfering effects makes the calculation of correction factors

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(Severinghaus and Ferrebee, 1950) impracticable. Synthetic standards (Chen and Toribara, 1953; MacIntyre, 1954; Teloh, 1958), with a composition similar to that of the solutions analysed, cannot be used when the composition of the materials is widely variable. Internal standards (Baker, 1955) are of only limited value because few sources of interference affect calcium emission and that of the internal standard proportionately.

Background correction (Vallee, 1954; Margoshes and Vallee, 1956) is made by subtracting readings taken at wavelengths near the analytical wavelength from those taken at the analytical wavelength. This accounts for direct interference, but indirect interference effects present much greater difficulty.

Radiation buffers (Severinghaus and Ferrebee, 1950; MacIntyre, 1957), which incorporate an excess of interfering ions, can control sources of indirect interference which have no increased effect above certain concentrations of the interfering substance or which have a plateau region over a restricted range.

In the determination of magnesium, anion interference has been controlled (West and Cooke, 1960; Fawcett and Wynn, 1961) by adding a large excess of ethylene diamine tetra-acetic acid (EDTA). West and Cooke (1960) found that EDTA could also be applied in this way to the determination of calcium but that the excess had to be very much greater, 160-fold instead of 10-fold. They used the disodium salt of EDTA, from which the high background emission is a serious disadvantage, particularly with instruments not equipped for automatic spectral scanning.

The standard addition method (Rothe and Sapirstein, 1955) may be used when the relationship between concentration and emission is linear, and sources of indirect interference have the same proportional effect upon standard calcium added to diluted samples as upon the calcium already present. The original concentration is thus calculated from the equation:

$$\frac{\text{calcium in sample}}{\text{calcium added}} = \frac{\text{emission by sample}}{\text{increase in emission due to calcium added}}$$

We investigated the use of radiation buffers and the standard addition procedure but, as reported below, neither was found satisfactory for our purpose.

Mitchell and Robertson (1936) reported that high concentrations of strontium released calcium emission from the inhibitory effect of aluminium. Willis (1960), measuring calcium by atomic absorption spectroscopy, used strontium chloride to control phosphate interference, and we tested its use for the same purpose in flame photometry. We also explored the potentialities of other compounds and achieved the best results with magnesium sulphate, which is readily available in a calcium-free state.

Since we completed our investigation on a method of releasing calcium emission from anion interference, Dinnin (1960) has reported results of an investigation on alkaline earth and rare earth elements, and iron, yttrium, and scandium as releasing agents, and he has proposed an explanation for their mode of action. The results of our investigation are reported below.

EXPERIMENTAL

INSTRUMENTAL The Unicam SP 900 flame spectrophotometer was used. It has a Meker type burner utilizing acetylene and compressed air. The latter conveys the atomized solution from a separate spray chamber. The instrument is equipped with a fused silica prism monochromator and a photomultiplier detector. A slit width of 0·08 mm., corresponding to a nominal band width of 1 mμ, was selected for all experiments. Air pressure was 30 lb./in.^{2} and acetylene pressure was about 12 cm. of water, except when the effect of altering flame conditions was being tested.

PREPARATION OF SAMPLES Samples were diluted to give a final calcium concentration of about 0·05 to 0·10 mM. Plasma samples were obtained from heparinized blood and diluted 1 : 50. Twenty-four-hour urine samples were preserved with 10 ml. of hydrochloric acid and diluted 1 : 50 or 1 : 100, according to the expected calcium concentration. Faeces were prepared by homogenizing with water and transferring portions of about 2 g. to nickel crucibles of 50 mm. diameter. The contents were dried at 105° and ashed overnight in the uncovered crucibles at 475 to 525°. If magnesium, as well as calcium, was to be determined, ashing was at 420 to 450° because of loss of magnesium at higher temperatures (Fawcett and Wynn, 1961). The ash was dissolved by careful boiling with 1 ml. of 1·0 N. hydrochloric acid, transferred quantitatively and diluted to 100 ml. This solution was further diluted 1 : 50 to 1 : 10, according to the expected calcium concentration.

GALVANOMETER READINGS When special diluting solutions were used, standard calcium solutions were diluted in the same diluent as the samples, and this diluent was also used for zero-setting the galvanometer. Blank and standard readings were checked between every two or three readings of unknowns. Each dilution was read at least three times, and more often if any reading differed by more than 1% from the mean.

INTERFERENCE BY SODIUM AND POTASSIUM The effects of sodium were observed by adding various concentrations of spectroscopically pure sodium chloride to deionized water and to solutions containing 0·05 mM and 0·10 mM calcium chloride.

Sodium interference was of two kinds: direct interference due to spectral radiation by sodium in the region of the calcium line, and indirect interference due to

enhancement of calcium emission by sodium. It was found that direct interference by sodium was proportional to its own concentration and represented about 0·04% of the emission from equimolecular concentrations of calcium, under the conditions of the investigation. Ten mM sodium chloride gave identical readings at 422·7 mμ, 418 mμ, and 428 mμ, and readings at the latter two wavelengths were unaffected by calcium emission.

In contrast to the direct interference, the enhancement interference at any stated sodium level was directly proportional to calcium concentration. As the sodium concentration was raised, the percentage enhancement rose to a maximum of 9% at 1 mM, remaining at this percentage for sodium concentrations at least as high as 10 mM. This is shown in Fig. 1.

The interference effects of potassium were similar to those of sodium, and direct interference was corrected in the same way. In the presence of 2 mM sodium chloride, as a radiation buffer, there was no further enhancement of calcium emission by added potassium up to 5 mM.

In subsequent investigations, direct interference was excluded by subtracting the mean of readings at 418 mμ and 428 mμ from readings at the calcium line, 422·7 mμ. Indirect interference by sodium and potassium was controlled by incorporating 2 mM NaCl in all standards and sample dilutions.

INTERFERENCE BY PHOSPHATE AND SULPHATE The effects of phosphate were observed by adding spectroscopically pure sodium dihydrogen phosphate to the solutions, and excluding the effects of sodium.

At any stated calcium concentration between 0·02 and 0·2 mM, the depression of calcium emission by phosphate was constant within the range 0·5 to 10 mM phosphate. The percentage depression of calcium emission by phosphate increased, however, with calcium concentration. Added phosphate thus distorted the linear relationship between calcium concentration and emission, as shown in Fig. 2. The degree of distortion varied with flame conditions, such as the pressures of air and acetylene, and it was difficult to reproduce exactly the same curve on different occasions.

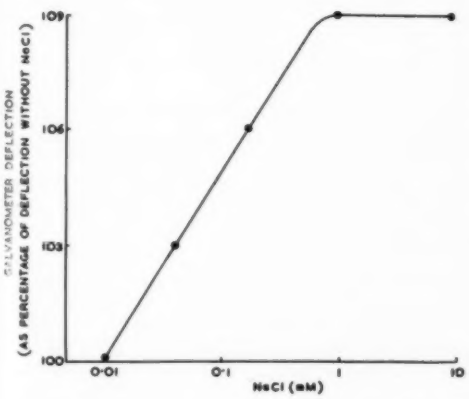

FIG. 1. *Enhancement of calcium emission by sodium.*

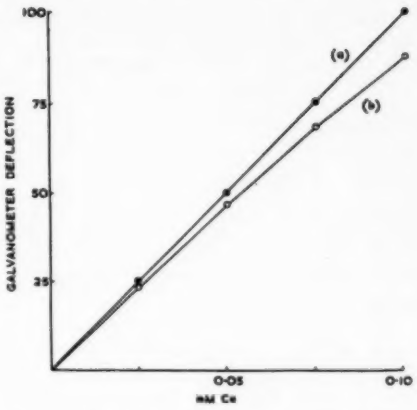

FIG. 2. *Depression of calcium emission by phosphate. Curve (a): Calcium emission in absence of phosphate. Curve (b): Calcium emission in presence of 0·5 mM NaH_2PO_4. Enhancement of calcium emission by Na was controlled by including 2 mM NaCl in all solutions.*

Like phosphate, 10 mM sulphuric acid or 10 mM ammonium sulphate caused depression of calcium emission and distortion of the calibration curve. In the presence of 10 mM sulphate, however, 1 mM phosphate had no additional effect on calcium emission. Similarly, in the presence of 0·5 mM sodium dihydrogen phosphate, as a radiation buffer, there was no further depression due to added sulphate up to 0·5 mM.

STANDARD ADDITION METHOD The standard addition method depends upon proportionality between calcium concentration and emission. As phosphate and sulphate caused distortion of the calcium calibration curve it was suspected that the method might not be valid when applied to biological materials. This was investigated as follows.

If the standard addition method were valid, the result calculated for any sample concentration should have been independent of the known quantity of standard calcium added. Therefore two different quantities were added to portions of three plasma samples and four urine samples diluted in water. The two results calculated for each sample were compared. These seven experiments were carried out on five different occasions, with slight variations in flame conditions.

Calcium concentrations were calculated as

$$2{\cdot}5\ \text{mM} \times \frac{E_0}{E_1 - E_0} \quad \text{(Formula 1)}$$

and

$$5{\cdot}0\ \text{mM} \times \frac{E_0}{E_2 - E_0} \quad \text{(Formula 2)}$$

where E_0 = emission by sample diluted 1 : 50 in water, E_1 = emission by the diluted sample incorporating 0·05

mM calcium chloride, and E_2 = emission by the diluted sample incorporating 0·10 mM calcium chloride.

Table I shows the results of these experiments. The two results for each sample concentration usually differed by 2 to 5 %. The non-linear relationship between calcium concentration and emission, in the presence of plasma or urine, is apparent from the last column of Table I. This shows that the increase ($E_1 - E_0$) in emission when the concentration was raised by 0·05 mM was usually substantially greater than the additional increase ($E_2 - E_1$) on raising the concentration by a further 0·05 mM. These findings were consistent with the earlier observations that, in the presence of phosphate, calcium emission was no longer proportional to concentration.

TABLE I

CALCULATION OF CALCIUM CONCENTRATION USING THE STANDARD ADDITION METHOD

Sample	*Ca Concentration of Sample (mM.)*		*Percentage Difference*	$\frac{E_1-E_0}{E_2-E_1}$
	Calculated from Formula 1	*Calculated from Formula 2*		
Plasma 1	1·74	1·74	0	1·00
Plasma 2	2·52	2·57	2	1·04
Plasma 3	2·72	2·85	5	1·10
Urine 1	0·32	0·34	6	1·11
Urine 2	0·55	0·56	2	1·03
Urine 3	2·30	2·38	3	1·08
Urine 4	5·00	5·33	7	1·13

CALCIUM RELEASE METHOD Phosphate interference in calcium emission was investigated with solutions diluted in 10 mM $SrCl_2$, 2 mM NaCl, and in 10 mM $MgSO_4$, 2 mM NaCl. Calcium emission was depressed by about 4% by 0·5 mM phosphate in the former diluent but was not depressed at all in the latter.

Table II shows the release of calcium emission from the depressing effect of phosphate when the diluent contained 10 mM magnesium sulphate. When the concentration of phosphate was raised above 2 mM, however, calcium emission decreased rapidly, and depression was very much greater than that caused by phosphate in the absence of magnesium sulphate. The concentration of phosphate which could be controlled varied with the concentration of magnesium sulphate. Ten mM magnesium sulphate was adequate to control phosphate interference at concentrations up to 100 mM in samples diluted 1 : 50, or up to 200 mM in samples diluted 1 : 100. Under the conditions of the investigation, the magnesium sulphate appeared to cause a 1% enhancement of the calcium emission.

With 2 mM sodium chloride in the diluent, sodium concentrations at least as high as 400 mM had no effect on calcium emission when samples were diluted 1 : 50. Table II also shows that hydrochloric acid depressed calcium emission by a maximum of 3%, but the effect was negligible at concentrations of 1 mM or less. This was about the concentration of hydrochloric acid in the final dilutions, when it was used as a urine preservative or to dissolve faecal ash.

TABLE II

EFFECT OF VARIATIONS IN COMPOSITION OF DILUENT ON EMISSION BY 0·10 mM $CaCl_2$

$MgSO_4$(mM)	*NaCl (mM)*	*NaH_2PO_4(mM)*	*Galvanometer Deflection*[1]
0	2	0	100
0	2	1	85
10	2	0·5	101
10	2	1	101
10	2	2	101
10	2	2·3	68
10	2	2·7	45
10	2	3	45
10	2	5	43
5	2	0	100
5	2	1	98
5	2	2	45
20	2	0	101
20	2	2	101
20	2	4	95
10	0	0	93
10	1	0	101
10	2	0	101
10	3	0	101
10	5	0	101
10	10	0	101
		HCl (mM)	
10	2	1	101
10	2	5	100
10	2	20	99
10	2	50	97
10	2	100	97

[1]As percentage of deflection with 0·10 mM $CaCl_2$ in 2 mM NaCl.

With 0·05 mM calcium, and the same concentrations of other solutes as those recorded in Table II, galvanometer deflections were one-half of those reported for

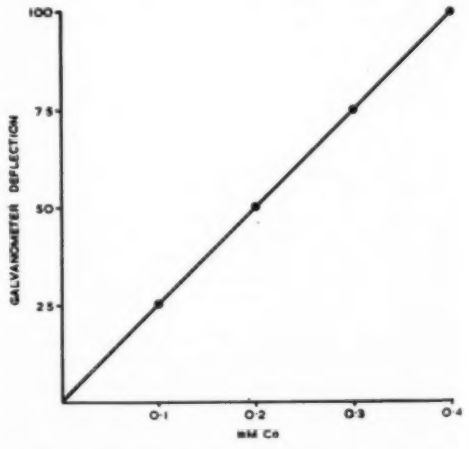

FIG. 3. *Calibration curve, using 10mM $MgSO_4$, 2 mM NaCl as diluting solution.*

0·10 mM calcium. The only occasion when calcium concentration and emission were not proportional was when phosphate was added in the absence of magnesium sulphate. In the presence of magnesium sulphate, however, even when phosphate concentration was so high that calcium emission was depressed, the percentage depression was the same at both calcium levels.

When a diluting solution containing 10 mM $MgSO_4$, 2 mM NaCl, was used, calcium emission was proportional to concentrations at least as high as 0·4 mM, the highest concentration which could be measured at the slit width of 0·08 mm. The calibration curve is shown in Fig. 3.

The background readings at 418 mμ and 428 mμ were usually about 2% of the readings at 422·7 mμ for plasma and urine, but were usually negligible for faeces.

PLASMA VISCOSITY The effect of the viscosity of 1 : 50 dilutions of plasma on the results obtained was tested by comparing the rate of uptake of diluted plasma into the atomiser with that of standard solutions, and by comparing calcium results obtained with and without prior ashing of the plasma. The rate at which 1 : 50 plasma dilutions were drawn into the atomiser was found to be 3% less than the uptake rate of standard solutions. When seven samples of plasma were analysed both directly and after ashing, calcium emission from the unashed samples was an average of 3·6% (S.D. 1·6%) less than the emission after ashing them.

The possibility that the lower surface tension of diluted plasma, compared with standard solutions, might affect the proportion of the aerosol reaching the flame was also investigated. A surface-active agent (Pluronic F 68) was added to a standard calcium solution to reduce the surface tension well below that of diluted plasma. Calcium emission in the flame, however, was not affected.

The physical properties of plasma dilutions thus depressed observed calcium results by 3 to 3·6%, with the instrument used by us. A 3% correction factor was selected and the validity of this is supported by the results of the recovery experiments reported below, in which this correction factor was used.

RESULTS

RECOVERY EXPERIMENTS USING CALCIUM RELEASE METHOD Recovery experiments were carried out on 16 samples of plasma, 15 of urine, and 14 of faeces. Samples were prepared as described above, incorporating 1 ml. of a stock solution of 0·5 M $MgSO_4$, 0·1 M NaCl, in each 50 ml. dilution. Dilutions were prepared in parallel, with the inclusion of known quantities of calcium. In the recovery experiments on faeces, calcium was added sometimes before ashing and sometimes afterwards, the results being reported separately. The experimentally determined viscosity factor was used in calculations of recoveries from plasma.

Results of the recovery experiments are reported in Tables III, IV, and V. Mean recoveries were

TABLE III

RECOVERIES OF CALCIUM ADDED TO 16 DILUTED PLASMA SAMPLES

Ca Added[1] *(mM)*	*Ca Recovered*[1] *(mM)*	*Percentage Recovery*
1·25	1·27	101·6
1·25	1·27	101·6
1·25	1·28	102·4
2·50	2·43	97·2
2·50	2·44	97·6
2·50	2·45	98·0
2·50	2·46	98·4
2·50	2·46	98·4
2·50	2·49	99·6
2·50	2·50	100·0
2·50	2·50	100·0
2·50	2·50	100·0
2·50	2·52	100·8
2·50	2·53	101·2
2·50	2·55	102·0
2·50	2·56	102·4
	Mean	100·1
	S.D.	1·9

[1]In terms of apparent increase in plasma Ca concentration.

TABLE IV

RECOVERIES OF CALCIUM ADDED TO 15 DILUTED URINE SAMPLES

Ca Added[1] *(mM)*	*Ca Recovered*[1] *(mM)*	*Percentage Recovery*
2·50	2·47	98·8
2·50	2·51	100·4
2·50	2·52	100·8
2·50	2·54	101·6
5·00	4·85	97·0
5·00	5·00	100·0
5·00	5·00	100·0
5·00	5·05	101·0
5·00	5·05	101·0
5·00	5·05	101·0
10·00	10·05	100·5
10·00	10·05	100·5
10·00	10·10	101·0
10·00	10·10	101·0
10·00	10·25	102·5
	Mean	100·5
	S.D.	1·3

[1]In terms of apparent increase in urine Ca concentration.

TABLE V

RECOVERIES OF CALCIUM ADDED TO (*a*) SEVEN SAMPLES OF FAECES BEFORE AND (*b*) SEVEN SAMPLES OF FAECES AFTER ASHING

Ca Added[1] *(m-mole)*	*Ca Recovered*[1] *(m-mole)*		*Percentage Recovery*	
	(*a*)	(*b*)	(*a*)	(*b*)
10·0	9·9	—	99·0	—
10·0	—	9·9	—	99·0
10·0	9·9	10·1	99·0	101·0
10·0	10·0	10·2	100·0	102·0
10·0	10·0	9·9	100·0	99·0
20·0	19·4	19·5	97·0	97·5
20·0	—	20·3	—	101·5
75·0	75·0	76·0	100·0	101·3
125·0	123·0	—	98·4	—
		Mean	99·1	100·2
		S.D.	1·1	1·6

[1]In terms of apparent increase in daily output.

100·1% for plasma, 100·5% for urine, 99·1% for faeces to which calcium was added before ashing, and 100·2% for faeces to which calcium was added after ashing. Standard deviations were less than 2%.

COMPARISON BETWEEN RESULTS OBTAINED BY CALCIUM RELEASE METHOD AND BY EDTA TITRATION The results of 26 calcium determinations on plasma, 10 on urine, and 11 on faeces obtained by flame photometry using the 10 mM $MgSO_4$, 2mM NaCl, diluent were compared with those obtained by titration with EDTA. The method for plasma was direct titration at *p*H 12 with automatic detection of the end-point (Wootton and Haslam, to be published).The method for urine and faeces was the back-titration of a measured excess of EDTA with a standard calcium solution at *p*H 12 using a calcein-thymolphthalein indicator.

Results of this comparison are recorded in Tables VI, VII, and VIII. Values for plasma were consistently 2% higher by flame photometry than by titration, but the differences with urine and faeces were small and unsystematic.

TABLE VI

COMPARISON OF RESULTS ON 26 PLASMA SAMPLES OF CALCIUM DETERMINATIONS BY FLAME PHOTOMETRY, USING THE CALCIUM RELEASE METHOD, AND BY EDTA TITRATION

Calcium Concentration (mM)		*Difference (mM)*
By Flame Photometry[1] *(a)*	*By EDTA (b)*	*(a−b)*
1·22	1·17	+0·05
1·37	1·32	+0·05
2·18	2·15	+0·03
2·32	2·27	+0·05
2·35	2·30	+0·05
2·35	2·30	+0·05
2·37	2·32	+0·05
2·38	2·33	+0·05
2·42	2·35	+0·07
2·43	2·32	+0·11
2·43	2·42	+0·01
2·45	2·39	+0·06
2·46	2·43	+0·03
2·49	2·44	+0·05
2·52	2·52	0
2·55	2·52	+0·03
2·55	2·55	0
2·62	2·60	+0·02
2·68	2·60	+0·08
2·69	2·62	+0·07
2·70	2·62	+0·12
2·76	2·72	+0·04
2·77	2·70	+0·07
2·82	2·75	+0·07
2·95	2·88	+0·07
3·46	3·42	+0·04
	Mean	+0·05
	S.D.	0·03

[1]3% was added to each observed result by flame photometry to correct for the effect of plasma viscosity.

TABLE VII

COMPARISON OF RESULTS ON 10 URINE SAMPLES OF CALCIUM DETERMINATIONS BY FLAME PHOTOMETRY, USING CALCIUM RELEASE METHOD, AND BY EDTA TITRATION

Calcium Concentration (mM)		*Difference (mM)*
By Flame Photometry (a)	*By EDTA (b)*	*(a−b)*
0·32	0·26	+0·06
0·40	0·44	−0·04
0·57	0·57	0
3·86	3·81	+0·05
3·93	4·05	−0·12
4·15	4·15	0
4·98	4·89	+0·09
4·94	5·00	−0·06
5·01	5·20	−0·19
7·61	7·81	−0·20
	Mean	−0·04
	S.D.	0·10

TABLE VIII

COMPARISON OF RESULTS ON 11 SAMPLES OF FAECES OF CALCIUM DETERMINATIONS BY FLAME PHOTOMETRY, USING CALCIUM RELEASE METHOD, AND BY EDTA TITRATION

Calcium Output (m-mole/day)		*Difference (m-mole/day)*
By Flame Photometry (a)	*By EDTA (b)*	*(a−b)*
1·5	1·6	−0·1
3·3	3·3	0
8·2	8·3	−0·1
9·0	8·7	+0·3
11·4	10·7	+0·7
14·7	14·0	+0·7
20·1	19·9	+0·2
20·2	20·3	−0·1
21·9	21·5	+0·4
63·0	61·5	+1·5
76·0	76·0	0
	Mean	+0·3
	S.D.	0·4

DISCUSSION

The proper control of interference effects in the determination of calcium in biological materials by flame photometry requires that each source of interference should be considered individually. Allowance can be made for differences in the physical properties of solutions and, when a monochromator is used, it is simple to correct for direct interference by subtracting background readings. Enhancement of calcium emission by cations can be kept constant by taking advantage of the plateau effect. Anions, such as phosphate and sulphate, which depress calcium emission, present a more difficult problem because of their variable influence at different calcium levels.

The effect of phosphate in distorting the linear relationship between calcium emission and concentration was noted by Jackson and Irwin (1957). The data of Baker and Johnson (1954) are difficult to interpret in this respect because, while their Fig. 2

shows that percentage depression by phosphate increased with increasing calcium concentration, at similar phosphate levels their Fig. 7 shows a linear relationship between calcium emission and concentration. Other workers (Chen and Toribara, 1953; Denson, 1954; Margoshes and Vallee, 1956; MacIntyre, 1957; Teloh, 1958) reported the effect of phosphate at a single calcium level and did not refer to distortion of the calibration curve.

Reports on the quantitative aspects of phosphate interference are generally conflicting. Although this might be attributed partly to the diversity of instruments and conditions used, it is noteworthy that Jackson and Irwin (1957), who observed non-proportionality of calcium concentration and emission in the presence of phosphate, employed a system different from our own, namely, a Beckmann DU spectrophotometer, a hydrogen-oxygen flame, and a wavelength of 554 mμ.

If the distorting effect of phosphate has sometimes been overlooked, this might help to account for the large discrepancies which have been found between results of flame photometry and of other methods, to which Hunter (1958) and Willis (1960) have drawn attention. Our findings agree well with those obtained by EDTA titration, even the 2% discrepancy with plasma being much less than that reported by others.

Of the known methods of controlling indirect interference effects in flame photometry, the use of radiation buffers or the addition of standard calcium to the samples, seemed to us the most hopeful. We found, however, that calibration curves using radiation buffers in the presence of phosphate were difficult to reproduce on different occasions, an effect which we attributed to variations in flame conditions. The standard addition method also proved unsound because of the non-proportionality of calcium concentration and emission in the presence of phosphate. Further disadvantages of this method were the superimposition of errors because of its dependence upon differences between large values, and the necessity to prepare a standard for each sample, even in a large series.

In the method presented here, calcium emission was released from depression by phosphate by the addition of a large excess of magnesium sulphate. This also eliminated any effect on calcium emission by the much smaller quantities of sulphate and magnesium ions already in the samples. When 10 mM $MgSO_4$, 2 mM NaCl was used for zero-setting and dilution of standards and samples, and when simple corrections were made for direct background interference and viscosity effects, the results obtained on biological materials were independent of variations in the concentration of interfering substances likely to be encountered.

Dinnin (1960) has published a comprehensive report on releasing effects in flame photometry. Although he used strontium and praseodymium and not magnesium salts in quantitative analysis for calcium, he drew attention to the potentialities of magnesium in this connexion, particularly because of its low background emission in the region of the calcium line.

Dinnin's explanation of the mechanism of releasing effects is based upon chemical equilibria in the evaporating droplets of solution, involving an exchange of anions between calcium and the releasing cation. Two of our observations seem inconsistent with this explanation. Although magnesium was added in the form of the sulphate, there was no depression of calcium emission. In the presence of 10 mM magnesium sulphate, there was a sudden and large depression of calcium emission when the concentration of phosphate was raised above 2mM, and this occurred at calcium concentrations of both 0·05 mM and 0·10 mM. This behaviour seemed more consistent with stoichiometric reactions involving magnesium and phosphate ions, in which calcium played no direct part, than with the displacement of equilibria. These theoretical aspects of calcium release are being investigated further.

The flame spectrophotometer used in this study was kindly lent to us by Unicam Instruments Ltd.

We thank Dr. P. H. Sanderson who carried out the EDTA titrations on plasma, Mr. P. B. Wells and Mr. H. Thomas who carried out the EDTA titrations on the samples of urine and faeces, and Mr. J. A. Corbett who drew our attention to the work of Dr. J. B. Willis on flame absorption spectroscopy.

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Estimation of gamma globulin in the serum of patients with hypogammaglobulinaemia

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SYNOPSIS Three methods of estimating γ globulin in the serum of patients with hypogammaglobulinaemia are described and compared, namely free electrophoresis, the inhibition of antiglobulin antiserum method, and the gel diffusion precipitin method. Data from 142 parallel estimations are reported.

The errors of these methods are determined.

The results of the three techniques give good general agreement. This is due to the fact that the 7S component dominates the estimations whichever technique is used. Some discrepancies are discussed.

All of the techniques are applicable to the diagnosis of hypogammaglobulinaemia. Their practical limitations are discussed.

In 1956, at the request of the Ministry of Health, the Medical Research Council established a working party to study hypogammaglobulinaemia, to organize the supply of γ globulin to patients with this condition in the United Kingdom, and to observe the effect of treatment. One of the criteria accepted for diagnosis was that the serum γ globulin concentration should be less than 200 mg./100 ml. (less than 100 mg./100 ml. for infants under 6 months old). To establish this and to observe the effect of the γ globulin injections on the serum γ globulin concentration of treated patients, three different laboratories made estimations of γ globulin by three different techniques. At the Lister Institute free electrophoresis was used, at the M.R.C. Blood Transfusion Research Unit the inhibition of antiglobulin serum, and at Birmingham University Medical School the gel diffusion precipitin technique.

The detailed techniques and the results of these estimations are reported here; we discuss the identity of the proteins estimated by the electrophoretic and the immunological techniques, the relative errors of these techniques, and their application to the diagnosis of hypogammaglobulinaemia.

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METHODS

Venous blood was collected by the physician in charge of the patient, and samples of the separated serum were sent to us by post. Since many of the patients suspected of having hypogammaglobulinaemia were children, the volume of serum was often small, and it was not always possible to estimate the γ globulin by all three techniques. We therefore report the results obtained on those sera which were tested by more than one technique.

ELECTROPHORESIS An accurately measured volume of serum, usually 4·0 ml., was pipetted into a cellophane membrane, and dialysed at 2°C. for a period of three days against 10 vol. of phosphate buffer (*p*H 8·0, I 0·2; Na_2HPO_4 0·326 M, KH_2PO_4 0·022 M), the buffer being changed six times during the course of the dialysis.

The dialysed serum was made up quantitatively with buffer to 10·0 ml. in a volumetric flask and the solution was cleared either by centrifuging, or, if very turbid, by passing through a clarifying filter in a centrifugal Seitz filter (M.S.E. Ltd.). The refractive index of the buffer (N_0) and of the serum protein solution (N_1) was determined at 25·0°C. with a dipping refractometer, using light of wavelength 546 mμ. isolated from a mercury arc with a suitable light filter. From the refractive index difference $N_1 - N_0$ the refractive increment N_s of the total protein in the undiluted serum was calculated by multiplying by the appropriate dilution factor.

For electrophoresis, the dialysed serum protein solution

was diluted with buffer to a refractive increment of 0·00350. It was subjected to electrophoresis in the long centre section cell type of Tiselius U-tube in a bath at 0·0 to 0·2°C. The potential applied was adjusted to give a current of 10·0 mA for the first hour of electrophoresis and then was increased to 15 mA and maintained at this level for a further three hours.

The diagonal Schlieren optical system (Philpot, 1938) was used to follow the course of electrophoresis and photographs were taken on Ilford half tone panchromatic plates with light of wavelength 546 mμ isolated from a mercury arc by a suitable filter.

The final pair of photographs obtained from the anode and cathode limbs of the U-tube after four hours of electrophoresis was projected with an optical enlargement of eight diameters onto graph paper and the curves drawn in. The curves were analysed geometrically by reflection and the areas corresponding with the individual components measured with a planimeter. From the results obtained the composition of the serum was calculated in terms of the percentage of the total refractive increment attributable to each component, taking the average of the values derived from the anode and cathode curves.

The refractive increment of the γ globulin in the serum is given by $N_s \times G \times 10^{-2}$ (where G is the percentage of N_s attributed to γ globulin). This value is converted in mg./100 ml. by dividing by 0·00189, the specific refractive increment of γ globulin (Perlmann and Longsworth, 1948) and multiplying by 1,000. Therefore the γ globulin concentration is given by:—

$$\frac{N_s \times G \times 10}{0{\cdot}00189} \text{ mg./100 ml.}$$

INHIBITION OF ANTIGLOBULIN SERUM The principle of this method is that γ globulin inhibits the reaction between an antihuman γ globulin serum and red cells sensitized with incomplete Rh-antibody (Coombs and Mourant, 1947). Very small amounts of globulin will inhibit the reaction (Wiener, Hyman, and Handman, 1949), and thus a test can be devised to estimate the serum γ globulin concentration in cases of hypogammaglobulinaemia (Wiener, 1955; Grubb, 1956; Mollison, 1956).

The technique used in this work was essentially that described previously (Mollison, 1956).

As a standard, a large sample of serum was obtained from a normal donor and stored at −20°C. Aliquots of this serum were thawed from time to time and a 1 in 2,000 dilution prepared by pipetting 0·5 ml. of serum with an Ostwald pipette into 1,000 ml. of saline. From this diluted serum, further dilutions of 1 in 3,000, 1 in 4,000, 1 in 5,000, 1 in 6,000, 1 in 7,000, 1 in 8,000, 1 in 9,000, and 1 in 10,000 were prepared, using relatively large volumes to minimize errors (*e.g.*, 5 ml. of 1 in 2,000 + 5ml. of saline to prepare a 1 in 4,000 dilution). The diluted sera were kept at +4°C. for not more than three months. (Sodium azide at a final concentration of 1 in 1,000 was added to the diluted sera to prevent the multiplication of bacteria.)

Red cells coated with γ globulin were prepared by incubating Rh-positive red cells for an hour with a serum containing incomplete anti-Rh, and then washing the cells three times in large volumes of a 1% saline solution. The same anti-Rh serum was used throughout the investigation.

Several different antiglobulin sera (prepared in rabbits) were used, but the dilution chosen for a particular estimation was always one which gave only moderately strong agglutination with the Rh-sensitized cells.

Serum from a suspected case of hypogammaglobulinaemia was tested as follows: the patient's serum was diluted in 1% saline to give concentrations of 1 in 50, 1 in 200, 1 in 800, 1 in 1,600, 1 in 3,200, and 1 in 6,400. One volume of diluted antiglobulin serum was placed in each of seven tubes and an equal volume of the 1 in 50 dilution of the patient's serum was added to the first tube; an equal volume of the 1 in 200 dilution was then added to the second tube, and this procedure was continued to provide seven mixtures containing a constant concentration of antiglobulin serum and decreasing concentrations of the patient's serum. A similar series of mixtures was prepared using the nine dilutions (1/2,000 up to 1/10,000) of the standard serum.

After 10 minutes, one drop from each mixture of the test series and of the standard series was placed in a separate square on a large opal glass tile. One drop of a 20% suspension of sensitized cells was added to each, and, after two minutes, and again after five minutes the strength of agglutination in each square was noted. The highest dilution of human serum giving a definite inhibition of the agglutination in each series was taken as the end-point.

Thus, if the end-point of the standard serum (which contained 1,200 mg. γ globulin per 100 ml.) was a dilution of 1 in 4,000, and that of the test serum was 1 in 50, the concentration of γ globulin in the test serum would be very approximately

$$\frac{50}{4{,}000} \times 1{,}200 = 15 \text{ mg./100 ml.}$$

After this preliminary test had been made, further dilutions of the test serum were prepared to define the γ globulin content more precisely. For convenience, these dilutions were prepared in an arbitrary series, *e.g.*, 1 in 200, 1 in 300, 1 in 400, 1 in 500, 1 in 600, 1 in 700, and 1 in 800. The difference between successive dilutions varied from about 15% to 50%. It was often possible to decide that the end-point fell between two dilutions; for example, the inhibition produced by a 1 in 600 dilution of test serum might be obviously greater than that produced by a 1 in 10,000 dilution of standard serum, whereas the inhibition produced by a 1 in 700 dilution of test serum might be obviously less. It would then be concluded that a 1 in 650 dilution of the test serum was equivalent to a 1 in 10,000 dilution of the standard serum in its ability to inhibit the reaction.

In reading the end-point, a series of comparisons was always made. For example, the dilutions of test serum and standard serum giving slight inhibition were noted and those giving almost total inhibition were noted. In this way, two or more estimates of the value were obtained, and the result was given as the mean of these values. It was found that the method could be made more sensitive by taking readings after the sensitized cells and anti-

globulin serum had been allowed to react for only about two minutes.

The γ globulin content of the standard serum was estimated at the beginning of the present work by free electrophoresis (Tiselius apparatus); the serum was found to contain 14·8% γ globulin (mean of ascending and descending boundaries); the total serum protein concentration was 7·8 g./100 ml. and the γ globulin content of the serum was therefore estimated at 1,155 mg./100 ml. After the serum had been stored for three years a second estimate was made by free electrophoresis (Perkins-Elmers instrument). The serum protein was found to contain 13·8% of γ globulin, a result in reasonable agreement with the first estimate. Results reported were based on the first (Tiselius) estimate of the standard.

GEL DIFFUSION PRECIPITIN The method, described by Gell (1957), entails visual matching of patterns of precipitate produced when suitable dilutions of unknown serum and a standard are allowed to react with a series of dilutions of a specific rabbit anti-human γ globulin antiserum, in agar gel.

Preparation of antiserum Sandy lop (M.R.C. strain) rabbits were immunized with purified γ globulin (fraction G3 of Kekwick and Mackay, 1954), kindly supplied by the Blood Products Laboratory, the Lister Institute, Elstree. A mixture of equal volumes of Freund's adjuvant (Freund and McDermott, 1942) and of a 1 g./100 ml. solution of γ globulin was emulsified and 1 ml. was injected subcutaneously at two different sites on both the first and the fourteenth day of the course. On the twenty-eighth day 0·5 ml. of the 1% γ globulin solution was injected intraperitoneally, and on alternate days thereafter increasing volumes up to 1 ml. (0·2, 0·5, 0·75, 1, 1, 1 ml., etc.) of the γ globulin solution was injected intravenously until the rabbits became dyspnoeic a few minutes after the injection. Five days after the last injection 60 to 80 ml. of blood was taken from the marginal ear vein. Rabbits which produced a satisfactory antiserum were reinjected every six months, using the intraperitoneal and intravenous course only.

The antisera were assessed by immunoelectrophoresis. There were a few weak contaminating antibodies in most of the antisera but, as the anti-γ globulin antibody was very strong, these did not produce lines of precipitation at the dilutions used for the estimations. As shown in Fig. 1, only one line of precipitation was seen when such a diluted antiserum interacted with serum from a patient with hypogammaglobulinaemia.

The standard was a preparation of purified γ globulin (fraction G3, see above) supplied by the Lister Institute, containing 8·4 g./100 ml. of γ globulin, as estimated by electrophoresis and the micro-Kjeldahl method. It was stored frozen in divided portions and was diluted to 1 g./100 ml. when required for use.

Davis New Zealand agar was dissolved in *p*H 7·4 phosphate buffer saline (K_2HPO_4 2·75%, KOH 0·8%, NaCl 1·7%), containing 1/10,000 sodium azide, by immersion in a boiling water bath for two hours, filtered through boiled filter paper pulp by suction, and 8 ml. poured into each flat Petri dish, 6·7 cm. internal diameter (depth of agar about 2·5 mm.). When the agar had set, a

FIG. 1. *Agar plate for gel diffusion precipitin technique.*

pattern of six large holes, each surrounded by six small holes (as illustrated in Fig. 1) was cut by a special cutter which removed the agar plugs by suction.

Dilutions were made by counting drops from a Pasteur pipette and a single drop of each dilution of antigen or antibody was placed in each hole in the agar plate. Dilutions of 1/50, 1/100, 1/200, and 1/400 of the standard 1% γ globulin were placed in four of the large holes, as shown in Fig. 1. Dilutions of the unknown serum of 1/8, and 1/64 were placed in the other two large holes (see Fig. 1). When the γ globulin content was very low, the estimation was repeated, using eight drops and one drop respectively of undiluted serum.

Six stock logarithmic dilutions of the antiserum were made. These varied with the antiserum, but a suitable series for a strong antiserum would be 1/8, 1/11, 1/15, 1/20, 1/26, 1/33. Single drops of these dilutions were placed in the small holes surrounding the antigen containing holes (marked a to f on Fig. 1). As shown in Fig. 1, each 'antigen hole' was surrounded by an identical series of six 'antibody holes' (though for economy's sake, the higher antiserum concentrations were shared by two antigen holes so that consecutive antigen holes were surrounded by a mirror image arrangement of antibody dilutions).

The Petri dish was covered, and left at room temperature for about 16 hours, while diffusion and precipitation occurred. A hexagon of precipitation developed around each antigen hole, the appearance of which depended solely on the concentration of γ globulin in the hole. It will be seen in Fig. 1 that the pattern of the precipitation around each of the 'standard holes' was clearly different from the others, that around the holes containing the higher γ globulin concentrations being nearer the antibody holes, and that around the lower concentrations being nearer the antigen hole. The lines also showed

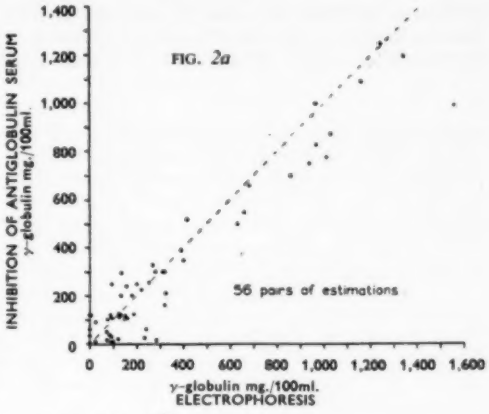
characteristic appearance at different concentrations, being either well defined and narrow at optimal proportions, or diffuse in antigen or antibody excess. The pattern around the holes containing the unknown serum could be matched line by line with those around the holes containing the standard. In Fig. 1, a 1/8 dilution of a hypogammaglobulinaemia serum matched precisely the 1/100 dilution of the 1 g./100 ml. standard, *i.e.*, 10 mg./100 ml. The 1/64 dilution of the unknown was correspondingly weaker than the 1/400 standard. Therefore the serum contained $8 \times 10 = 80$ mg./100 ml. Such direct matches in this plate would give readings of 20, 40, 80, 160, 320, 640, and 1,280 mg./100 ml. By using one drop and eight drops of undiluted serum, this series could be continued down to 0·3 mg./100 ml. Interpolations could also be made, when the unknown appeared midway between two standards, or rather nearer one or another. Results of such interpolations were expressed as arithmetic intermediate steps, *e.g.*, *20*, 25, 30, 35, *40*, 50, 60, 70, *80*, 100 mg./100 ml. etc. (the direct matches are in italics).

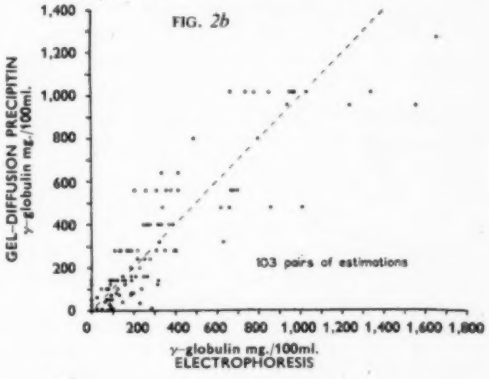
RESULTS

The results of estimations of γ globulin in 142 serum specimens are represented graphically in Fig. 2. These include all samples submitted for diagnostic purposes, and subsequent samples obtained during the course of treatment from 35 patients in whom the diagnosis was confirmed. A wide range of γ globulin concentrations was measured, from less than 1 mg. to nearly 2 g./100 ml. The higher figures were obtained from suspects in whom the diagnosis was not confirmed, and from treated cases. All specimens were estimated by the gel diffusion precipitin method, but only some of them by the other two methods. The points representing specimens estimated by all three techniques are plotted in closed circles, and those estimated by only two by open circles.

There is an obvious positive correlation between each set of results, and the points appear reasonably consistent with the 45° line marked on each graph. The two graphs which include the gel diffusion precipitin results have a large scatter at higher values, as would be expected from a titration technique using a geometric scale of dilutions. The two graphs, which include the electrophoresis results, have a larger scatter at the lower levels (below about 300 mg./100 ml.), as compared with the plot of the two immunological techniques which precisely approaches the origin.

DETERMINATION OF ERRORS OF TECHNIQUES The error of each technique was estimated. A series of five dilutions of normal human serum was made, using hypogammaglobulinaemia serum as diluent, so that they covered most of the range of the unknown sera.





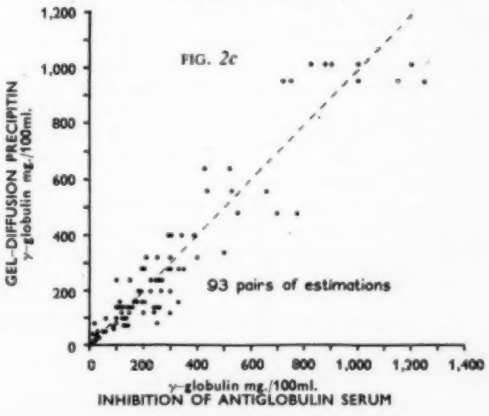


FIG. 2c
GEL-DIFFUSION PRECIPITIN γ-globulin mg./100ml.
93 pairs of estimations
γ-globulin mg./100ml. INHIBITION OF ANTIGLOBULIN SERUM

FIG. 2. *Plots of the estimations of γ globulin in the serum from the patients suspected of, or under treatment for, hypogammaglobulinaemia by each of the three techniques used. The dotted line is drawn at 45°.*

Each was estimated five times by each of the two immunological techniques. The results are given in Table I. For the gel diffusion precipitin technique,

TABLE I

RESULTS OF EXPERIMENTS TO DETERMINE ERRORS OF TWO IMMUNOLOGICAL TECHNIQUES FOR ESTIMATING SERUM γ GLOBULIN OVER A WIDE RANGE OF γ GLOBULIN CONCENTRATIONS

Method	*Mean Concentration (mg./100 ml.) (Five Determinations)*	*Standard Error (mg./100 ml.)*	*Coefficient of Variation (%)*
Gel diffusion precipitin	1,120	63	14
	496	28	13
	144	8	12
	44	2·5	12
	8·4	0·5	11
Inhibition of anti-globulin serum	2,174	128	8
	1,012	59	6
	516	30	18
	156	9	18
	93·4	5·5	11

the coefficients of variation throughout the range of concentration of γ globulin were constant and a value pooled from all the data was 13%. For the inhibition of antiglobulin serum technique, the coefficient of variation does not vary consistently with the concentration; the pooled value is 13%.

Unfortunately, there was not enough material available for a similar study of the error of electrophoretic measurements to be made. However, four estimations were made on a pool of normal serum and on each of a series of mixtures of purified albumin and γ globulin (fractions AP3 and G4 of Kekwick and Mackay, 1954). The albumin concentrations of these mixtures were maintained constant at a level corresponding to that obtained when normal human serum was diluted for electrophoretic examination, as described previously. The γ globulin content of the mixtures was varied to correspond with serum contents of 50, 100, and 200 mg./100 ml. The results are shown in Table II.

These errors show no consistent relationship to concentration but the scatter shown in Fig. 2 is larger than could be expected from the application

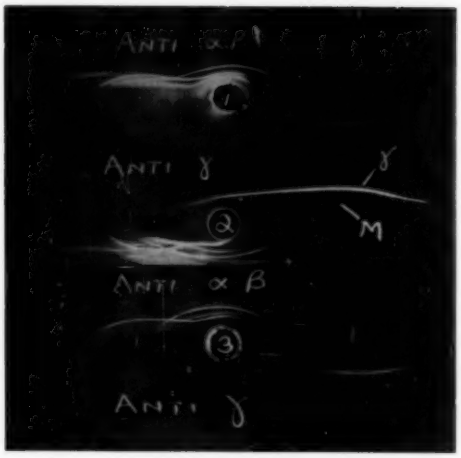


FIG. 3. *Immunoelectrophoresis of serum from a healthy adult male (2) and from two patients with hypogammaglobulinaemia, one probably of the congenital type (1) and one probably of the acquired type (2) reacting with a rabbit anti-human γ globulin antiserum typical of the ones used for the gel diffusion precipitin technique, and with a rabbit antiserum containing antibodies against many human globulins, but with little or no anti-albumin or anti-γ globulin. The γ globulin precipitin line (labelled γ), elongated as compared with those due to the other serum proteins in normal serum, is deficient in sera 1 and 3. The line of the 19S component of γ globulin (identical with the β_2M of continental workers), labelled M, which is shown by both antisera, is present in sera 2 and 3, but absent in serum 1.*

of these estimates. It seems likely, therefore, that there is a difference in the nature of the material estimated by each of the techniques.

NATURE OF PROTEIN ESTIMATED BY EACH TECHNIQUE

Gamma globulin is defined as the slowest moving component observed during the electrophoresis of human serum by the moving boundary method at slightly alkaline *p*H values. It is definitively estimated from the area of the peak produced by the application of Schlieren optical methods or by

TABLE II

RESULTS OF EXPERIMENTS TO DETERMINE ERROR OF ESTIMATION OF γ GLOBULIN BY ELECTROPHORESIS

Material	*Approximate γ Globulin Concentration (mg./100 ml.)*	*γ Globulin (% of total protein) (mean of four determinations)*	*Standard Error*	*Coefficient of Variation*
Normal human serum	1,000			1·6
γ globulin and albumin mixtures	200	3·8	0·30	16
	100	2·2	0·25	23
	50	0·9	0·08	17

interferometric procedures. Other methods of estimating it must clearly be substantiated by comparison with electrophoretic estimations.

In patients with hypogammaglobulinaemia, the substance of practical importance is antibody protein, and though this is known to be associated predominantly with γ globulin in man, there is evidence to suggest that antibody activity may be associated with components of greater electrophoretic mobility. Gamma globulin is electrochemically heterogenous; moreover, it includes a protein whose sedimentation coefficient is 19S, as well as the predominant 7S component.

The electrochemical heterogeneity of γ globulin is well illustrated by immunoelectrophoresis. In Fig. 3, the anti γ globulin antiserum used for the gel diffusion estimations and an antiserum against a wide range of human α and β globulins are shown interacting with normal human serum and with sera from two patients with hypogammaglobulinaemia. The γ globulin precipitin line in normal serum is longer and straighter than the precipitin lines for other proteins, most of which are symmetrical arcs (Grabar and Burtin, 1960). This results from the fact that γ globulin molecules have a wide range of electrophoretic mobility. It will be seen that there is material reacting with the same antibody stretching well forward into the β and α positions. This material is not γ globulin, as defined electrophoretically above, but it may be antibody protein. There is no doubt that the gel diffusion precipitin technique estimates all of the protein thus antigenically defined.

The 19S component of the electrophoretically defined γ globulin is antigenically distinct from the 7S component, and though antibody to it is present in some of the antisera used for estimating γ globulin by the gel diffusion technique (see Fig. 3), this would form a separate precipitin line and would be diluted out, so that it does not affect the estimation of the 7S component. Specific antisera to this protein (also called β_2M), which is often deficient in serum of patients with hypogammaglobulinaemia (Gitlin, Hitzig, and Janeway, 1956), can be used to estimate it by the gel diffusion technique (see later).

The specificity of the inhibition of antiglobulin serum technique does not depend on the specificity of the antiserum used, which may contain antibodies against many human serum proteins. The specificity of the test depends upon the fact that only protein which is antigenically identical with Rh-antibody protein will inhibit the reaction between antiglobulin serum and Rh-sensitized red cells. There is evidence that incomplete anti-Rh is a 7S γ globulin (Fudenburg, Kunkel, and Franklin, 1959) and it seems likely that the interaction of this protein with anti-γ globulin will be inhibited by all the 'γ globulin' demonstrated by immunoelectrophoresis to interact with antibody to 7S γ globulin (see Fig. 3).

Probably therefore the two immunological techniques are estimating the same substance, that is to say, all the protein of the line marked 'γ' in Fig. 3. As already mentioned, this includes some protein with the mobility of α and β globulins which will not be included in an electrophoretic estimate of γ globulin. On the other hand, it will not include 19S γ globulin, marked 'M' in Fig. 3, some of which will be included in an electrophoretic estimate.

Slater (1955) estimated the γ globulin content of samples of human serum by a precipitin method using an anti-γ globulin serum and found that with normal sera and sera containing an abnormally large amount of γ globulin, the immunological estimates were 20 to 40% higher than electrophoretic estimates on the same samples. This presumably represented the difference between total '7S antibody protein' (immunological technique) and electrophoretically defined γ globulin, *i.e.*, part of the 7S + part of the 19S. There is, however, no direct evidence that the whole of the proteins defined antigenically as '7S' is potential antibody or is, in fact, 7S.

In view of Slater's findings, the apparent agreement in the present work between estimates by immunological techniques and by electrophoresis needs explanation. So far as the inhibition of the antiglobulin serum method is concerned, the explanation seems to be provided by the fact that whole human serum rather than γ globulin was used as a standard, and it was in fact shown that if γ globulin were used as a standard, the 'γ globulin' content of normal human serum was overestimated. Thus Table III shows the results of tests on three normal sera and two preparations of purified γ globulin. Using one of the γ globulin preparations as a standard, the amount of γ globulin in the other γ globulin preparation was correctly estimated. But estimates for the γ globulin concentration of the three normal sera were all far too high in comparison

TABLE III

γ GLOBULIN CONCENTRATION OF THREE NORMAL SERA AND ONE PREPARATION OF γ GLOBULIN (LKG.11) USING ANOTHER γ GLOBULIN PREPARATION (G.4) AS A REFERENCE STANDARD

Sample	*Estimate of γ Globulin Concentration (mg./100 ml.)*	
	Inhibition of Anti-globulin Serum	*Free Electrophoresis*
Normal serum (M.C.)	2,020	1,155
Normal serum (J.F.S.)	1,270	970
Normal serum (G.C.J.	1,640	1,270
γ globulin (LKG. 11)	900	930

with the estimates obtained by electrophoresis (see Table III). Thus agreement with electrophoresis is obtained by this technique only when whole serum, which has had its γ globulin content defined by electrophoresis, is used as a standard. Where electrophoretically faster moving antibody protein and the 19S component of γ globulin vary *pari passu* with the 7S component agreement with electrophoresis will he obtained. When a purified γ globulin standard is used the result presumably represents total 7S antibody protein.

For the gel diffusion precipitin technique purified γ globulin was used as a standard and it is therefore difficult to see why estimates obtained with this technique were not greater than those obtained by electrophoresis.

Immunoelectrophoresis shows that in hypogammaglobulinaemia sera the whole of the 7S antibody protein line is deficient; in fact there is only a faint line visible with a mobility corresponding to the centre of the line present in normal sera, that is, to the position where the concentration is usually greatest. Therefore, a discrepancy between the techniques due to the electrochemical heterogeneity of γ globulin is likely to be in the direction of a systematic bias rather than an increased scatter of results.

Nevertheless, a discrepancy between electrophoresis and immunological estimates could arise from variations in the relative amounts of other proteins, *e.g.*, 19S γ globulin, if this does not vary proportionally with 7S γ globulin. This situation was observed in three patients in our series, and it may well explain some of the excessive scatter in the results obtained in parallel estimations of the sera from individual patients by the electrophoretic and the immunological techniques.

Estimation of the serum γ globulin of two of these (G.B. and N.B.) by electrophoresis gave consistently higher results than by the two immunological techniques, as shown in Table IV. And in the third (C.B.) paper electrophoresis of serum gave an apparently normal γ globulin band, though the gel diffusion precipitin method showed grossly depleted γ globulin. Thus in all these three cases electrophoretic estimates were higher than those obtained by immunological techniques. It was shown that these three cases were the only ones (out of the 41 studied) in whose sera the 19S component of γ globulin was not greatly reduced in concentration. It has previously been shown by immunochemical methods that the 19S component of γ globulin is one of the other two serum proteins besides the main 7S γ globulin component which are deficient in most patients with hypogammaglobulinaemia (Gitlin *et al.*, 1956). In two of these three patients, the 19S component was estimated by the gel diffusion precipitin technique only, but in the third an ultracentrifugal analysis was also made. The serum of patient N.B. had a normal concentration of 50 mg./100 ml.; that of G.B. had an abnormally high concentration of 100 mg./100 ml.; C.B. had the remarkably high concentration of 500 mg./100 ml. This boy (C.B.) who will be reported in detail elsewhere (Bodian, Marshall, and Weston, 1961) presented with *Pneumocystis carinii* pneumonia which led to the suspicion of hypogammaglobulinaemia. On paper electrophoresis the γ globulin concentration was normal, but an estimate by the gel diffusion precipitin technique gave a result of only 35 mg./100 ml. Ultracentrifugal analysis showed that there was a normal 4·5S, but no 7S component detectable and also confirmed that the 19S component was increased (670 mg./100 ml.). This includes the 19S α_2 glycoprotein of presumably normal concentration (about 150 mg./100 ml.). Unfortunately, there was not enough serum for Tiselius electrophoresis but an estimate by paper electrophoresis (kindly performed by Dr. J. Hardwicke) gave a figure for the γ globulin concentration of 960 mg./100 ml. The excess 19S component accounts for much of the discrepancy, which would probably have arisen also with Tiselius electrophoresis. The 7S component of γ globulin is often low in macroglobulinaemia, but this patient showed no other features of this disease. The 'γ globulin' by electrophoresis on paper was indistinguishable from that of a normal child of this age.

TABLE IV

RESULTS OF ESTIMATIONS OF γ GLOBULIN ON SERA FROM TWO PATIENTS WITH HYPOGAMMAGLOBULINAEMIA

Patient	*Date*	*Electrophoresis (mg./100 ml.)*	*Inhibition of Antiglobulin Serum (mg./100 ml.)*	*Gel Diffusion Precipitin (mg./100 ml.)*
G.B.	19. 9.56	285	17·5	10
	19.12.56	315	300	120
N.B.	25. 1.57	233	25	35
	14. 3.57	320	160	140
	25. 4.58	400	—	280

APPLICATION TO THE DIAGNOSIS OF HYPOGAMMAGLOBULINAEMIA Some γ globulin was detected in the serum of all 61 patients with hypogammaglobulinaemia studied by the M.R.C. working party, so that a quantitative estimate was required for making the diagnosis. In one, no γ globulin was detected by the gel diffusion precipitin technique, for which the lowest limit of detection is 0·06 mg./100 ml. Using the inhibition of antiglobulin serum method, slight but definite inhibition was obtained

with the patient's undiluted serum and, by comparison with suitable dilutions of whole human serum, it was estimated that the γ globulin concentration was about 0·1 mg./100 ml.

Techniques routinely available at present in laboratories for estimating γ globulin are the zinc sulphate precipitation method (Kunkel, 1947) and paper electrophoresis. These are not sensitive at low concentrations of γ globulin. The zinc sulphate method gives little precipitation at the normal concentrations, and is really suitable only for abnormally high γ globulin concentrations. Paper electrophoresis estimation of the electrophoretic protein fractions has an error of about 6% of the total protein concentration (Hardwicke, 1954). In the sera studied this would amount to about 360 mg./100 ml., so that it is unsatisfactory for estimating γ globulin at concentrations less than 200 mg./100 ml. It is, however, a useful screening procedure. Provided that cases in which it may be misleading, such as C.B. described above, are borne in mind, an obvious and distinct γ globulin band on paper electrophoresis usually excludes the diagnosis of hypogammaglobulinaemia. But any serum in which the γ globulin band is at all indistinct requires investigation by other techniques.

Our results show that any one of the techniques studied by us is satisfactory for routine clinical use for the diagnosis of hypogammaglobulinaemia. However, they are not all practicable for a routine laboratory.

Electrophoresis has the advantage of being independent of standards. But, at low concentrations, it is not as sensitive as the other methods, and a single estimation takes a day to perform and requires an expensive apparatus not generally available.

The antiglobulin inhibition technique is probably the most sensitive of the methods studied. Moreover, all the reagents needed were readily available and almost any antiglobulin serum is suitable. On the other hand, the method is somewhat time-consuming, a batch of up to four estimations taking about three hours.

The gel diffusion precipitin technique, because of its logarithmic error (the standard deviation is about 13% of the concentration), is relatively insensitive at high concentrations, though this is no disadvantage when estimating a protein deficiency. It is quick and easy and is economical of the antiserum, which need not be completely specific.

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The antithrombin activity of glucuronic esters of bilirubin

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SYNOPSIS The plasma of patients with pronounced jaundice as well as that of cats with experimental obstructive jaundice inhibits the clotting of normal plasma with thrombin. The influence of different bile constituents—bilirubin, bilirubin glucuronides, sodium salts of bile acid—on the clotting of plasma or fibrinogen with thrombin was examined. Bilirubin glucuronides in concentrations above 8 mg. % showed antithrombic activity but other substances were inactive. Glucuronic acid and phenolphthalein glucuronide in concentrations up to 100 mg. % did not have any influence on the thrombin time.

It is well known that a prolongation of the plasma thrombin time can be observed in the course of liver disease associated with jaundice. According to Ratnoff (1953) and Alagille and Soulier (1957), the prolonged thrombin time in liver disorders is due to a deficiency of some factor in plasma rather than to the appearance of inhibitory substances. Ratnoff's (1953) view is that in plasma there is a co-factor of thrombin-fibrinogen interaction which is defined as 'thrombin accelerator', and its amount may be decreased in liver disease. Other authors suggested an increase of antithrombin activity in the plasma of these patients (Schwarz, Wanner, and Koller, 1951; Sokal, Schmid, and Hörder, 1955). Masure (1956) postulated qualitative modifications in fibrinogen molecules resulting in an increased resistance of fibrinogen to the action of thrombin.

We have found that the plasma of patients with pronounced jaundice, as well as that of cats with experimental obstructive jaundice, inhibits the clotting of normal plasma with thrombin, and the purpose of this paper is to examine the influence of bile constituents on the thrombin-fibrinogen interaction *in vitro* because it has been found that bilirubin glucuronides exhibit antithrombic activity.

METHODS AND MATERIALS

Blood from patients and from normal subjects was collected into 0·1 M ammonium oxalate in the proportion of one part of anticoagulant to nine parts of blood. Plasma was obtained by centrifuging for 10 min. at 1,500 r.p.m.

Cats' plasma was obtained in the same manner from the animals with experimental obstructive jaundice produced by ligating the choledochus duct and from control cats.

Fibrinogen was prepared from bovine plasma by the method of Blombäck and Blombäck (1957).

Thrombin was purchased from Hoffman la Roche and Co.

Bilirubin, glucuronic acid, phenolphthalein glucuronide, sodium salts of choleic, and desoxycholeic and glicocholic acid were commercial preparations. As a solvent for bilirubin, 0·1 M Na_2CO_3 was used.

Bilirubin glucuronides were prepared by the method of Cole, Lathe, and Billing (1954) from urine, blood, and bile from human patients with liver disorders. The identity of ester preparations obtained was confirmed by paper chromatography (Schmid, 1957), absorption spectra (Cole *et al.*, 1954), and the direct van den Bergh reaction. The preparations were protein free and freely soluble in water.

Paalisch buffer was made up of solutions A and B.

A Sodium borate 0·05 M 19·108 g. $Na_2B_4O_7$ 10 H_2O. . . . . 1,000 ml.

B Boric acid solution in saline, 12·404 g. H_3BO_3, 2·925 g, NaCl, distilled water to 1,000 ml.

and 1·2 vol. of A was mixed with 8·8 vol. of B. The final *p*H should be 7·6.

This buffer was used as a diluent for all reagents unless otherwise stated.

METHODS

The thrombin time was determined as follows:—

Plasma, 0·2 ml., or 0·2% fibrinogen was mixed with 0·2 ml. of the substance to be tested or the solvent used

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to prepare the solution of the above substance, and added to 0·1 ml. thrombin (diluted in order to obtain a clotting time of 10 to 15 sec. in the control system). Prothrombin time was determined by the method of Quick (1935) using rabbit brain thromboplastin.

The recalcification time was tested after adding 0·2 ml. plasma to the same volume of 0·025 M $CaCl_2$.

The inhibitory influence of bile constituents on the prothrombin and recalcification times was determined after adding one volume of the substance to be tested to one volume of plasma.

The concentration of bilirubin and bilirubin glucuronides in plasma samples and in the preparations of bilirubin esters was determined by the method of Malloy and Evelyn (1937) using a Coleman D 206 B spectrophotometer.

RESULTS

ANTITHROMBIC ACTIVITY OF ICTERIC PLASMAS We have observed a markedly prolonged thrombin time in some patients with pronounced jaundice. Patients' and normal plasmas were mixed in various proportions and the thrombin time was determined in each mixture. A significant inhibitory influence of icteric plasma can be seen from the curve shown in Fig. 1. The same phenomenon was observed when the mixtures of normal and icteric plasma of cats were examined (Fig. 2).

INFLUENCE OF BILIRUBIN, BILIRUBIN GLUCURONIDES, GLUCURONIC ACID, PHENOLPHTHALEIN GLUCURONIDES, AND SODIUM SALTS OF BILE ACIDS ON THROMBIN TIME The influence of all these substances on clotting of normal plasma and on fibrinogen with thrombin was examined (Figs. 3 and 4). It was found that bilirubin up to a concentration of 200 mg.%[1] and sodium salts of bile acids up to the concentration of 100 mg. %[1] did not influence the thrombin time. On the contrary, bilirubin glucuronides showed a significant inhibitory influence in concentrations above 8 mg. %[1]. Therefore we examined glucuronic acid and phenolphthalein glucuronide and found that these substances in concentrations up to 100 mg. % did not have any influence on thrombin time.

THE INFLUENCE OF BILIRUBIN GLUCURONIDES ON PROTHROMBIN AND RECALCIFICATION TIMES It can be seen from Table I that the thrombin time of plasma and of fibrinogen as well as of the prothrombin and recalcification times of plasma are prolonged in the presence of bilirubin glucuronides.

[1]The values refer to the initial concentrations of solutions which were examined.

DISCUSSION

It appears that bilirubin glucuronides inhibit thrombin-fibrinogen interaction, while other tested bile constituents are not active. We were not able to confirm the finding quoted by Scheraga and Laskowski (1957) concerning the inhibition of fibrinogen-fibrin conversion by bile acids.

The possibility cannot be excluded that the different reactivity of bilirubin and its esters is related

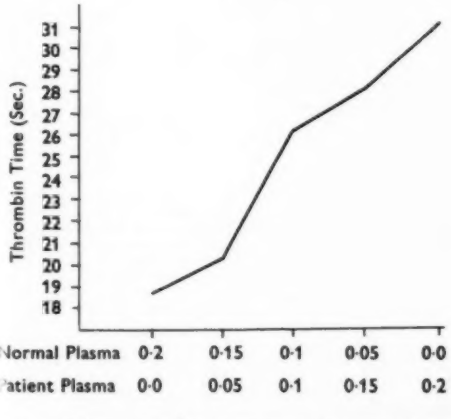

FIG. 1. *Influence of icteric plasma on the thrombin time of normal plasma. Patient was suffering from obstructive jaundice due to cholelithiasis. Level of total bilirubin in serum is 51 mg.% and of bilirubin glucuronides 40·3 mg.%.*

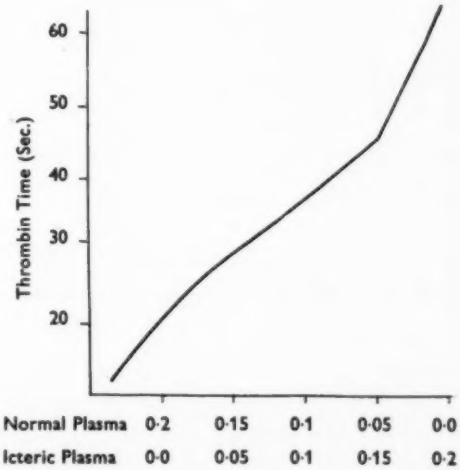

FIG. 2. *Influence of icteric cat plasma on the thrombin time of normal cat plasma. Level of total bilirubin in serum 21·7 mg.% and of bilirubin glucuronides 17 mg. %.*

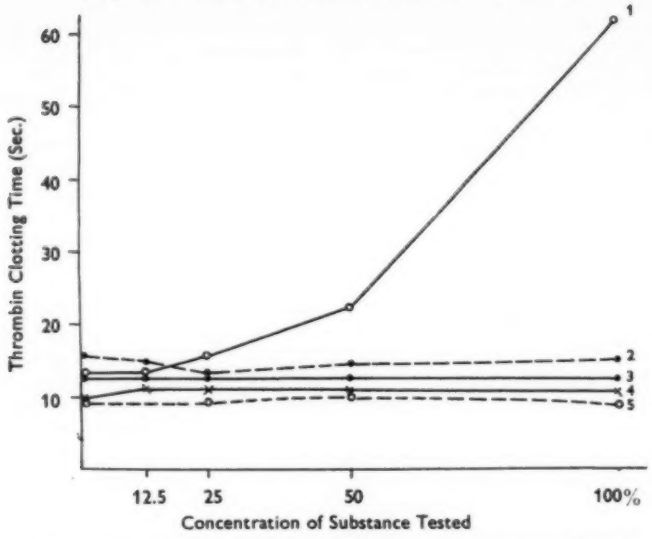

FIG. 3. *Influence of free bilirubin, bilirubin glucuronides, glucuronic acids, phenolphthalein glucuronides and bile acid salts on the clotting time of normal plasma with thrombin. Initial concentrations: Bilirubin esters, 17·5 mg. %; glycocholic acid, 20 mg. %; free bilirubin, 200 mg. %; glucuronic acid, phenolphthalein glucuronide, sodium glycocholate, sodium deoxycholate, sodium salt of choleic acid, 100 mg. %; and cholic acid, 25 mg. %.*

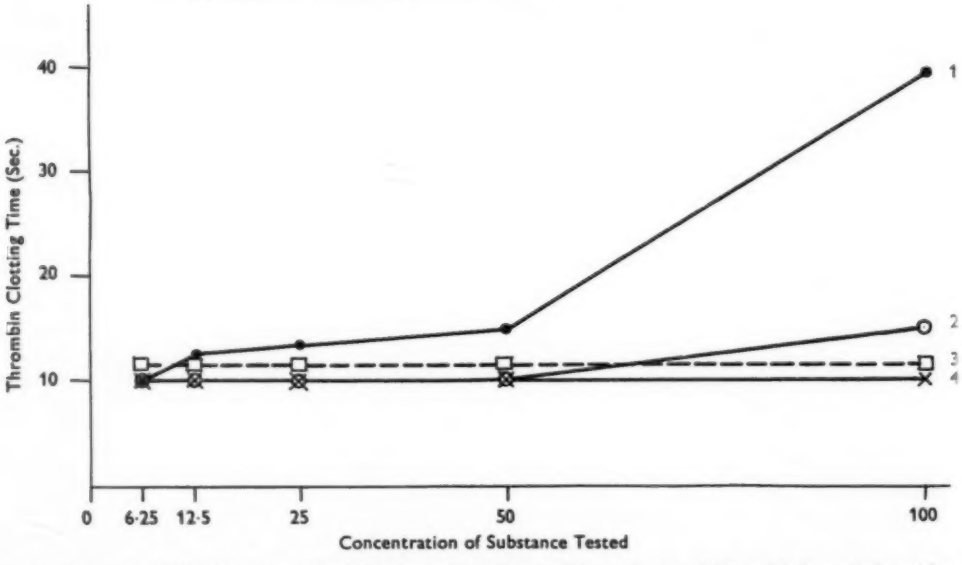

FIG. 4. *Influence of bilirubin esters, free bilirubin, sodium salt of cholic acid, glycocholic acid, deoxycholic acid, and glucuronic acid on clotting time of 0·2% fibrinogen with thrombin. Initial concentrations: Bilirubin esters, 19 mg. %; free bilirubin, 200 mg.%; sodium glycocholate, sodium deoxycholate, glucuronic acid 100 mg.%; and sodium salt of cholic acid, 100 mg.%.*

TABLE I

INFLUENCE OF BILIRUBIN GLUCURONIDES ON THROMBIN, PROTHROMBIN, AND RECALCIFICATION TIMES

Concentration of Bilirubin-glucuronides Solution (mg.%)	*Thrombin Time (sec.)*		*Prothrombin Time (sec.)*	*Recalcification Time (sec.)*
	Plasma	*0·2% Fibrinogen*		
25	40	70	30	360
12·5	17	16	23	220
6·25	12	11	21	200
3·12	11	10	19	170
Buffer	11	11	18	150

to differences in solubility of these substances in plasma protein solutions, or to a decrease in the level of free bilirubin in the presence of plasma protein.

Further studies are necessary to elucidate the detailed mechanism of the inhibitory action of bilirubin glucuronides.

The prolongation of the thrombin time in liver disorders may well be of a complex nature and due to several factors. An increase in fibrinolytic activity of blood in hepatic patients may result in the appearance of the fibrinogen digestion product antithrombin VI (Niewiarowski and Kowalski, 1958; Kowalski, 1960). The dysproteinaemia, particularly low albumin level and high γ globulin level favour a prolongation of the thrombin time (Jim, 1957).

A raised level of bilirubin glucuronides could be one of the most specific factors influencing the thrombin time in the course of hepatic jaundice.

Measuring the prothrombin time is considered to be one of the most sensitive liver tests, for it permits the evaluation of the ability of liver parenchyma to synthesize plasma clotting factors. (It should be noted that the high levels of bilirubin glucuronides may interfere with the prothrombin time.) It is possible that the high level of bilirubin glucuronides in the plasma of icteric patients may be a contributory factor in the development of a haemorrhagic tendency.

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Studies on the disturbance of glucuronide formation in infectious hepatitis

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SYNOPSIS The ability of the liver to form glucuronides was measured in 10 patients with infectious hepatitis. One test was done at the onset and another about four weeks later after the clinical symptoms had disappeared. N-acetyl-p-aminophenol (N.A.P.A.) or acetanilide was administered in doses ranging from 10 to 20 mg. per kg. body weight, either orally or by intravenous injection. N.A.P.A. is conjugated by the liver at the hydroxyl group and excreted in the urine as sulphuric and glucuronic acid conjugates. Total conjugated p-aminophenol, free N.A.P.A., and N.A.P.A. glucuronide were estimated in the urine of our patients. In the blood the disappearance of N.A.P.A. (free form) and the formation of N.A.P.A. glucuronide were traced.

During the acute phase of hepatitis the excretion of total conjugated p-aminophenol and of N.A.P.A. glucuronide in the urine is lower than after recovery from the disease. Likewise free N.A.P.A. disappears more slowly from the circulation and the peak concentration of N.A.P.A. glucuronide in the serum remains lower at the onset of hepatitis than after clinical cure. These results indicate that glucuronide formation during the acute stage of infectious hepatitis is depressed, as are other transformation mechanisms, *i.e.*, of hippuric acid.

Studies of the directly reacting bile pigments either by reversed phase column chromatography (Billing, 1955a) or by partition between different solvents (Schachter, 1959); Eberlein, 1960) have given some indications of a disturbance of glucuronide formation in hepatitis. In the initial stages of viral hepatitis, apart from free bilirubin, pigment II, which is identical with bilirubin diglucuronide (Billing, Cole, and Lathe, 1957), is often present in the serum in increased amounts. As hepatic cells progressively degenerate, the less polar pigment I (bilirubin monoglucuronide) almost always forms the major bile pigment component (Billing 1955b; Bollman, 1959). Studies by Bollman (1957) in hepatectomized dogs or after poisoning the liver of experimental animals by carbon tetrachloride showed that bilirubin monoglucuronide can be formed outside the liver, but bilirubin diglucuronide is formed only in the liver. The increased amounts of pigment I in cases of hepatitis, therefore, suggest the presence of a secondary disturbance in the liver, so that the conjugation of bilirubin with glucuronic acid or the conversion of bilirubin monoglucuronide to the diglucuronide is incomplete (Billing and Lathe, 1958). Other transformation reactions, for instance, the conjugation of benzoic acid with glycine to form hippuric acid, are also decreased in hepatitis. It seemed justified therefore to explore further the possibility of a disturbance in glucuronide formation during hepatitis in a more direct way than is possible by investigating serum bilirubin fractions.

There are many substances besides bilirubin which are conjugated with glucuronic acid in the liver so as to make them more soluble and enhance their excretion. We chose N-acetyl-p-aminophenol (N.A.P.A.) for our tests. The use of a compound, which, after conjugation in the liver, is excreted in the urine, seemed advantageous in as much as its behaviour is not influenced by a disturbance of bile secretion. Such a disturbance without doubt exists during hepatitis and plays a role in the alterations of bilirubin metabolism.

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METHODS

Ten children with infectious hepatitis aged 2½ months to 15 years were studied twice during the course of their illness. One test was made at the beginning of hepatitis, in the first week after the appearance of symptoms. The

test was repeated about four weeks later, after clinical cure and after other liver function tests gave normal results. Six patients were given intravenous injections of 10 to 20 mg. of N.A.P.A./kg. weight dissolved in distilled water at temperature 60°C. and sterilized in an autoclave at 120°C. for 12 minutes. The solution was kept at 37°C. until use. The other four patients received 10 to 20 mg. of acetanilide per kg. body weight orally. Acetanilide is rapidly oxidized to N.A.P.A., which is then conjugated in the liver at the hydroxyl group. As will be shown, this hydroxy-conjugate is present in the serum almost exclusively in the form of N.A.P.A. glucuronide. However, apart from a small fraction of free N.A.P.A., it appears in the urine as N.A.P.A. glucuronide and -sulphate (Greenberg and Lester, 1946). After administration of the test substance urine was collected for a period of 24 hours in two separate 12-hour periods. Blood samples were drawn after 15 minutes and after one, two, three, four, six, or seven and sometimes nine hours and permitted to clot. Urine and serum were analysed for the free N.A.P.A., total conjugated p-aminophenol (consisting of N-acetyl-p-aminophenol and hydroxy-conjugated N-acetyl-p-aminophenol) and N.A.P.A. glucuronide. Free N.A.P.A. and total conjugated p-aminophenol were estimated by the methods of Brodie and Axelrod (1948). N.A.P.A. glucuronide was determined by measuring the amount of N-acetyl-p-aminophenol liberated from serum or urine by the action of bacterial-β-glucuronidase. Enzymatic hydrolysis was carried out by adding 10 mg. of bacterial-β-glucuronidase (Sigma Chemical Co.) to 1 ml. of serum or suitably diluted urine in 4 ml. of 0·15 M acetate buffer, pH 6·2, and incubated at 37°C. for 18 hours. The amount of N.A.P.A. glucuronide can be calculated by measuring free N.A.P.A. before and after hydrolysis (Vest and Streiff, 1959). The difference between total conjugated p-aminophenol and the sum of free N.A.P.A. and N.A.P.A. glucuronide represents N.A.P.A. sulphate.

In some cases the results were checked by independent estimation of glucuronides (Dische, 1947) and of sulphates (Folin, 1905) in the urine. The amounts of glucuronic acid and sulphate in the urine during the test period were compared with the average excretion of these substances during a control period, immediately before the administration of the test substance. Other liver function tests were done by standard methods.

RESULTS

EXCRETION OF N.A.P.A. METABOLITES IN THE URINE In Table I the excretion of total conjugated p-aminophenol in the early stages of hepatitis and after recovery is listed as a percentage of the dose of acetanilide or N.A.P.A. given. In all cases investigated total excretion was diminished during the acute phase. On the average it amounted to 60% during hepatitis and to 74·4% after cure. The latter value is identical with the average excretion found in normal children or adults. Only in early infancy is the percentage excretion lower because of immaturity of the liver (Vest, 1958). This is reflected in the low values found in the case of the 2½-month-old infant. There is no difference in the percentage excretion between the lower (10 mg. per kg. body weight) or higher (20 mg. per kg. body weight) dose of N.A.P.A. or acetanilide.

In column 5 of Table I the result of the first test is expressed as a percentage of the values found after recovery. From this it appears that the excretion during the acute phase of hepatitis was on the average only 81% of that reached after cure. Calculation of the significance of the difference between the two tests using the t test of Fisher (1954) shows that the observed decrease in percentage excretion is statistically highly significant, P being $<0{\cdot}001$.

The individual amounts of free N.A.P.A., of N.A.P.A. liberated by β-glucuronidase, and of other hydroxyconjugates (N.A.P.A.-sulphate) are listed in Table II for the six patients in whom these N.A.P.A. metabolites were individually estimated in the urine. The values given refer to the first 12-hour period after injection of N.A.P.A. They are expressed as a

TABLE I

EXCRETION OF TOTAL P-AMINOPHENOL AT ONSET OF HEPATITIS AND AFTER RECOVERY

Name	*Age*	*Substance and Dosage (mg./kg.)*	*Percentage Excretion in 24 Hours*		*Results of First Test as % of Second Test*
			During Hepatitis (First Test)	*After Recovery (Second Test)*	
B.G.	2½ mth.	Acetanilide 10	40	46	87
C.C.	5 yr.	Acetanilide 10	54·6	62·5	87·7
S.N.	10 yr.	Acetanilide 20	75	85	88·2
F.R.	3½ yr.	Acetanilide 20	42	63·5	66·1
R.M.	15 yr.	N.A.P.A. 10	73	86·6	84·3
T.A.	7 yr.	N.A.P.A. 10	51·5	81·5	63·2
M.H.	10 yr.	N.A.P.A. 10	73	93·3	78·3
K.V.	12 yr.	N.A.P.A. 20	64·5	84	77
K.A.	14 yr.	N.A.P.A. 20	71·7	80·4	89·1
N.C.	5 yr.	N.A.P.A. 20	55	61	90
Average			60·0	74·4	81·1

TABLE II

EXCRETION OF FREE N.A.P.A., N.A.P.A. GLUCURONIDE, AND N.A.P.A. SULPHATE DURING 12-HOUR PERIOD AFTER INJECTION OF N.A.P.A. AS PERCENTAGE OF AMOUNT ADMINISTERED

Name	*Age*	*Dosage of N.A.P.A. (mg./kg.)*	*Percentage Excretion in 12 Hours*					
			N-acetyl-p-aminophenol		*N.A.P.A. Glucuronide*		*N.A.P.A. Sulphate*	
			First Test	*Second Test*	*First Test*	*Second Test*	*First Test*	*Second Test*
T.A.	7 yr.	10	3·0	6·2	4·1	39·0	34·7	25·5
M.H.	10 yr.	10	3·7	5·4	24·2	43·1	43·5	40·8
R.M.	15 yr.	10	2·5	2·8	28·0	50·7	35·8	20·5
N.C.	5 yr.	20	3·7	3·3	19·2	24·3	16·1	26·4
K.V.	12 yr.	20	4·5	4·0	33·8	39·5	19·6	35·5
K.A.	14 yr.	20	4·0	3·0	40·5	37·8	20·8	32·1
Average			3·6	4·1	25·0	39·0	28·5	30·0

percentage of the dose administered. It is evident that in contrast to the overall excretion of Table I there is some difference according to the dose used in testing the formation of conjugates. With regard to the excretion of N.A.P.A. glucuronides, all patients with one exception, K.A., excreted more N.A.P.A. as glucuronide in the second test than during the acute phase. Even in the case of K.A., the glucuronide content was higher in the second test, when the whole 24-hour urine specimen is considered instead of a 12-hour specimen. But the increase of N.A.P.A. glucuronide in the urine after recovery from hepatitis is distinctly higher in the groups of patients receiving 10 mg. per kg. body weight of N.A.P.A. than in the three patients who were tested with 20 mg. per kg. Because the sulphate conjugates are calculated as the difference between total excretion and the sum of free N.A.P.A. and N.A.P.A. glucuronide, they tend to show an inverse relationship to the glucuronides. Therefore, N.A.P.A. sulphate forms a bigger part of the total conjugates in the group receiving 20 mg. N.A.P.A. per kg. than in the group with 10 mg. per kg. Likewise in the individuals who received 20 mg. per kg., N.A.P.A. sulphate showed an increase after recovery, whereas in the group with only 10 mg. per kg. it tended to be reduced. No persistent trend is evident in the excretion of free N.A.P.A., which rarely exceeds 5% of the dose administered. On the average of all patients, free N.A.P.A. increased from 3·6% to 4·1%, N.A.P.A. glucuronide from 25% to 39%, and other conjugates from 28·4% to 30%. Only the increase of the glucuronides is statistically significant ($P = 0.05$).

DISAPPEARANCE RATE OF FREE N.A.P.A. AND FORMATION OF N.A.P.A. GLUCURONIDE IN BLOOD At the beginning of the investigation it was presumed that in the blood, as in the urine, N.A.P.A. conjugates would consist of glucuronides and sulphates. However, a comparison between the total amounts of N.A.P.A. (estimated as p-aminophenol) in the same serum sample after acid hydrolysis by hydrochloric acid at 120°C. for one and a half hours and after enzymatic hydrolysis by β-glucuronidase showed no differences within the limits of accuracy of the chemical estimation. The results of these measurements are shown in Table III. Considering the comparatively low absolute concentration of p-aminophenol per millilitre serum, the agreement between the values found by the two different modes of hydrolysis is

TABLE III

COMPARISON BETWEEN ESTIMATIONS OF TOTAL N.A.P.A. (FREE AND CONJUGATED) IN SERUM AFTER ACID HYDROLYSIS BY CONCENTRATED HCL AND AFTER ENZYMATIC HYDROLYSIS BY β GLUCURONIDASE[1]

Name	*Acid Hydrolysis*					*Hydrolysis by β glucuronidase*				
	Hours					*Hours*				
	1	*2*	*4*	*7*	*9*	*1*	*2*	*4*	*7*	*9*
W.K.	18·0	19·0	16·6	8·4	4·3	13·5	19·0	16·7	5·7	3·9
M.H.	13·4	14·4	11·7	6·5	3·7	12·5	15·0	11·5	5·9	3·0
H.C.	33·4	31·5	25·4	13·3	9·1	39·0	33·0	24·5	13·8	9·5
B.D.	21·0	22·0	—	10·5	6·0	23·0	22·0	—	10·0	5·0
B.H.	31·0	25·0	18·0	6·5	4·5	29·5	25·0	14·8	5·0	4·0
G.S.	28·0	24·0	13·0	4·5	2·1	26·0	23·0	13·0	5·0	2·8
D.S.	23·0	18·5	15·5	5·5	1·9	26·6	22·0	16·0	7·0	2·0
Average	23·9	22·0	16·7	7·8	4·5	24·3	22·7	16·1	7·5	4·3

[1]Values are expressed in μg./ml.

obvious. From this it would appear that there is no other hydroxy-conjugate than glucuronide present in the serum. Contamination of the enzyme preparation with sulphatase, which at the same time would liberate sulphate conjugates, was ruled out. On the other hand, it is known that sulphuric acid conjugates of phenol and aminophenol are easily split by dilute acids (c.f. Levvy and Storey, 1949). Because acid is used in the procedure for the estimation of free N.A.P.A., the possibility exists that sulphate conjugates were measured along with free N.A.P.A. Studies are under way to investigate this question further. The same studies made with urine reveal the presence of a considerable amount of N.A.P.A. conjugates, which are not liberated by β glucuronidase, and on examination by the method of Folin (1905) were found to be bound to sulphate. This confirms the findings of Greenberg and Lester (1946) and others. The tests done with conjugates in the urine can be regarded as further proof against the presence of sulphatases in the enzyme preparation used. There remains the question how and where the N.A.P.A. glucuronide in the serum is converted in part to N.A.P.A. sulphate. It seems likely that the kidney might play a role in this transformation.

It can be seen from the examples shown in Figs. 1 to 4 that there is a distinct difference in the speed at which the N.A.P.A. concentration declines in the blood. During the acute phase the decrease of free N.A.P.A. is slower than after recovery from hepatitis. Likewise the level of N.A.P.A. glucuronide during the disease remains below the one attained after cure. In Fig. 1 (an example of an oral test done with acetanilide) the peak concentration of free N.A.P.A. is reached later than in the case of oral or intravenous N.A.P.A. because the substance has first to be oxidized in the para position. As a consequence, conjugation to the glucuronide sets in later. But basically the same trend is evident as in the examples 2, 3, and 4, when N.A.P.A. was administered orally or intravenously. During the acute phase the concentration of N.A.P.A. in the serum declines more slowly and the curve of N.A.P.A. glucuronide is flatter than in the same children after recovery.

The high immediate peak value for N.A.P.A. glucuronide shown in Fig. 2 may well be due to more rapid conjugation when the N.A.P.A. is absorbed directly into the portal circulation than when administered intravenously: on the other hand, as wide variations in the rate of conjugation and the levels reached have been observed in different subjects, this cannot be regarded as proved.

In Table IV the amount of free N.A.P.A. present six hours after the intravenous injection of N.A.P.A., or seven hours after the oral administration of N.A.P.A. or acetanilide is expressed as a percentage of the N.A.P.A. level at the beginning of the test, or in the cases of oral administration, of the maximal concentration of N.A.P.A. reached during the experiment. With one exception, where there was no significant difference between the results of both tests, the percentage retention of N.A.P.A. was always higher in the acute phase than in the second study done after liver function had returned to normal. In Table IV the maximum concentrations of N.A.P.A. glucuronide in μg./ml. are listed separately for the two studies done on each patient. Again, with the exception of the child who showed no difference in the percentage of N.A.P.A. retained, all the patients had higher values during the second test, *i.e.*, the curve of N.A.P.A. glucuronide in the serum took a flatter course during hepatitis than it does normally.

TABLE IV

RETENTION OF FREE N.A.P.A. IN SERUM DURING AND AFTER HEPATITIS

Name	*Age (yr.)*	*Dose Administered (mg./kg. body weight)*	*% Retention After Six and Seven Hours*		*Peak Level (μg./ml.) of N.A.P.A. Glucuronide in Serum*	
			First Test	*Second Test*	*First Test*	*Second Test*
		N.A.P.A. intravenously				
T. A.	7	10	14	11·9	1·6	4·5
M. H.	10	10	3·2	4·7	5·4	3·9
R. M.	15	10	26·8	8·4	4·5	6·0
N. C.	5	20	9·5	8·5	7·4	7·8
K. V.	12	20	14·8	11·6	6·0	7·7
K. A.	14	20	16·5	8·0	6·6	9·1
		N.A.P.A. orally				
G. S.	7	20	11·7	5·8	10·2	15·0
		Acetanilide orally				
F. L.	3½	20	54	21	1·7	7·1
S. L.	10	20	26	9·5	1·8	17·2

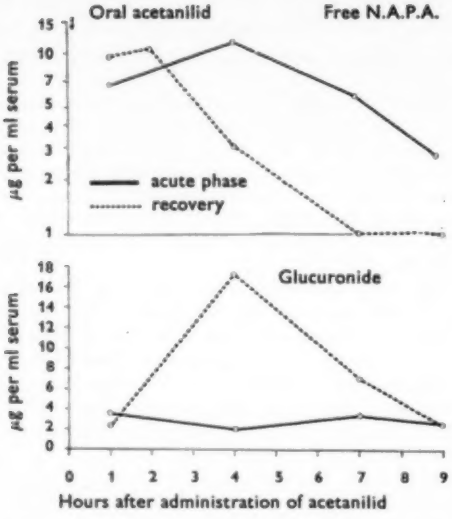

FIG. 1. *S.N., aged 10 years. Disappearance of free N.A.P.A. and formation of N.A.P.A. glucuronide in the serum during hepatitis and after recovery. Oral administration of acetanilide, 20 mg. per kg. body weight.*

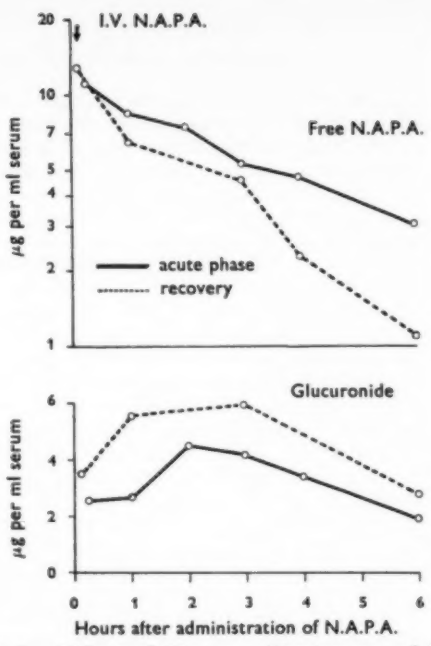

FIG. 3. *R.M., aged 15 years. Disappearance of free N.A.P.A. and formation of N.A.P.A. glucuronide during hepatitis and after recovery. Intravenous injection of N.A.P.A., 10 mg. per kg. body weight.*

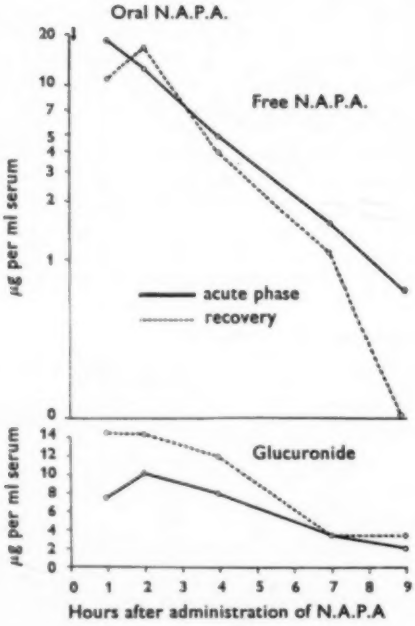

FIG. 2. *G.S., aged 7 years. Disappearance of free N.A.P.A. and formation of N.A.P.A. glucuronide in the serum during hepatitis and after recovery. Oral administration of N.A.P.A., 20 mg. per kg. body weight.*

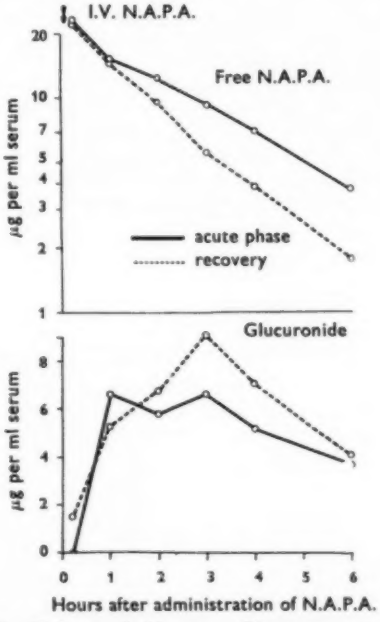

FIG. 4. *K.A., aged 14 years. Disappearance of free N.A.P.A. and formation of N.A.P.A. glucuronide in the serum during hepatitis and after recovery. Intravenous injection of N.A.P.A., 20 mg. per kg. body weight.*

DISCUSSION

In these studies we have shown that the urinary elimination of N.A.P.A. or acetanilide is diminished during viral hepatitis. The reduction in excretion of a given dose amounted on the average to 20% of the values found in the same individuals after recovery, with a variation from 10% to 37%. This reduction is reflected also in the values found for individual conjugates. The glucuronides, *i.e.*, that part of the hydroxy conjugates liberated by the action of β glucuronidase, decreased, especially during hepatitis, and showed a subsequent increase. Less consistent was the behaviour of sulphate conjugates. A distinct increase was seen only in the patients who received a dose of 20 mg. per kg. body weight of N.A.P.A. but not in the group receiving 10 mg. per kg. No clear trend was discernible with regard to free N.A.P.A. in urine, though on theoretical grounds one could perhaps have expected an increase of free N.A.P.A. when the conjugation mechanism is disturbed. Such an increase was seen only in the three cases receiving 20 mg. per kg. body weight of N.A.P.A. and even in these free N.A.P.A. contributed only a small percentage of the total.

The observed reduction in the excretion of a load of N.A.P.A. may result from a disturbance of the conjugation at the hydroxyl group. Even though we know of no reports of disturbance of kidney function in hepatitis, a failure of the kidneys to excrete the substance might, in principle, also cause a reduction in percentage excretion. If conjugation is disturbed one would expect a lower disappearance rate of free N.A.P.A. from the blood because of diminished hepatic transfer and low levels of N.A.P.A. conjugates. A diminished excretion on the other hand would lead to an accumulation of the conjugated product. An examination of N.A.P.A. and its metabolites in the serum would permit a distinction between these possibilities.

From the examples given in Figs. 1 to 4 and from the values in Table IV it can be seen that such a reduction in the disappearance rate of free N.A.P.A. is observed in the patients tested. Furthermore, glucuronide levels were lower than in the same individuals after recovery. From this a diminished renal clearance during hepatitis can be ruled out. There seems to be no doubt that the reduction in excretion of N.A.P.A. and the low levels of N.A.P.A. conjugates observed during hepatitis are the results of a disturbance in glucuronide formation. This then from a different approach confirms the findings of Billing (1955b), Bollmann (1959), and others of a decreased formation of bilirubin diglucuronide. The findings are also consistent with the well-substantiated fact of a diminished conjugation with glycine, as in hippuric acid formation (Quick, 1933) and in other transformation reactions (Snapper and Saltzman, 1949) during hepatitis.

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Rapid incremental methods for the determination of serum iron and iron-binding capacity

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SYNOPSIS Rapid methods depending on differential absorptiometry are described for the determination of the transferrin iron content and the latent iron-binding capacity of blood serum. Each determination requires as little as 0·5 ml. serum. The methods are well adapted for routine use in the 'average' laboratory. Three or four sera may be completely analysed in 30 minutes. All operations are carried out in the cells or tubes used for the colorimetric measurements, no precipitation or heating being employed at any stage. Critical investigations of the reliability of the methods are attempted and ranges of normal values are included.

The growing interest in iron metabolism and transport in man has created the need for methods for the determination of serum iron and iron-binding capacity which can be used routinely in the average clinical laboratory with the minimum expenditure of time, reagents, and equipment. The number and various types of method published indicate the value of iron analyses as aids in the diagnosis and treatment of various blood dyscrasias and abnormalities in iron metabolism, as typified by iron-deficiency anaemias, haemochromatosis, and the ageing process.

Earlier work on serum iron determination has been reviewed by Hemmeler (1951), Laurell (1952), and Ramsay (1958). Most methods involve the use of acid media to precipitate protein and to extract the iron, a procedure which was introduced by Barkan (1927). Some workers use heat to assist liberation of the iron, but others omit this. Methods involving wet ashing for determining total iron have been used to some extent but are tedious and time-consuming. Recently, Webster (1960) introduced a rapid method which omits protein precipitation but involves heating with a detergent. In nearly all cases the iron is complexed with thiocyanate, α, α′-dipyridyl, *o*-phenanthroline, bathophenanthroline or its sulphonate, and measured colorimetrically.

Most methods for the determination of latent iron-binding capacity depend on the small increase in light absorption at about 520 mμ when ferrous iron is added to the serum, either incrementally or in excess (Rath and Finch, 1949; Cartwright and Wintrobe, 1949; Ventura, 1952; Kaldor, 1955). The disadvantage of this kind of method is its low sensitivity, although some variations are simply and rapidly performed. In another type of method, excess iron is added to saturate the transferrin, the residual iron being removed by first complexing with *o*-phenanthroline. Strong acids are then added to liberate the serum-bound iron but not that complexed to *o*-phenanthroline, the latter being precipitated along with the serum proteins. A frequent problem with this type of method is the difficulty in obtaining an optically clear protein-free filtrate or supernatant; this is essential to the success of the method as even slight amounts of turbidity lead to erroneous results; the use of bathophenanthroline or its sulphate would reduce these errors somewhat by increasing the ratio of colour to turbidity but they would remain appreciable. Several blanks are required and the procedure is somewhat protracted.

The objectives of the present work were to develop rapid direct methods which avoid precipitation of proteins and heating, and require only small volumes of serum and simple blanks. The procedures to be described are carried out entirely in the optical cells or tubes used for the light absorption measurements, each determination requiring 0·5 ml. serum if bathophenanthroline or its sulphonate (Case, 1951; Peterson, 1953; Trinder, 1956) is used, or 1 ml. if *o*-phenanthroline is used as complexing agent.

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METHOD I:

SERUM TRANSFERRIN IRON DETERMINATION

PREPARATION OF GLASSWARE All glassware is completely wetted internally with the acid cleaning mixture described below and then washed in tap water and finally ion-free water. Apparatus is stored away from dust. Apart from the optical cells and serum pipettes, rinsing of glassware with ion-free water after use is all that is required.

COLLECTION OF BLOOD An iron-free syringe with a new needle is satisfactory. Specimens may be collected at any time, but where possible, early morning ones from fasting donors are preferable. The blood is stored in an iron-free tube. Plasma is unsatisfactory for the methods described.

REAGENTS These were made up as follows:—

Heated sodium dithionite 3% *in* 0·1 *M sodium acetate* Dissolve the solid in the acetate solution and heat about 20 ml. solution for five minutes in a boiling water bath. Cool the solution to room temperature. Use fresh preparations each day. (For determinations with *o*-phenanthroline the same reagent is used.)

Sulphonated bathophenanthroline, 0·4% To 200 mg. bathophenanthroline in a hard-glass boiling tube add 1 ml. chlorsulphonic acid and boil the contents gently over a small flame for 30 seconds. Cool the tube to room temperature and cautiously add 10 ml. iron-free water, followed by 6·5 ml. 5 N sodium hydroxide. Mix and heat in boiling water for a few minutes until a clear solution is obtained. Cool to room temperature and adjust the *p*H to 8 to 12 units. Add 0·5 ml. glacial acetic acid and mix. The *p*H should then be 4 to 5 units. Dilute to 50 ml. with iron-free water. Add a few drops of chloroform and keep the reagent refrigerated. This reagent is a modification of that described by Trinder (1956).

o-Phenanthroline, 0·25% Dissolve 125 mg. hydrated solid in 2·5 ml. alcohol and some water and dilute to 50 ml. (This reagent is an alternative to the preceding one.)

Standard iron solution, 0·5 mg. Fe/ml. Dissolve 0·3511 g. ferrous ammonium sulphate hexahydrate, A.R., in 0·1 N sulphuric acid (iron-free) and dilute to 100 ml. with this acid. This reagent is stable in total iron for a long time, although ferric ions slowly accumulate by aerial oxidation.

Standard iron solutions (dilute) Prepare a 100 ml. volumetric flask as follows. Treat the flask with the acid cleaning solution described below and wash thoroughly with tap water. Wet the whole of the inside of the flask with 2% aqueous cetyl trimethylammonium bromide (Cetavlon), wash thoroughly, and rinse with ion-free water. The detergent coats the glass and inhibits adsorption of iron. (If the detergent treatment was omitted it was found that the weak standards deteriorated with time.)

Measure 5 or 10 ml. stronger iron standard into the flask, add about 10 mg. ascorbic acid and dilute to the mark with iron-free water. These standards contain 25 or 50 µg. Fe/ml. respectively. The weaker one is used with bathophenanthroline or its sulphonate. Prepare them freshly about once a fortnight. Under these conditions all the iron is in the ferrous state.

Acid cleaning solution Dissolve about 100 mg. potassium permanganate in 200 ml. cold concentrated sulphuric acid. On no account should heat be applied because of the risk of explosive decomposition; the cold solution has been used for many years by one of us. Glassware is cleaned in the usual way and drained as free as possible of surplus water before treatment with this reagent, which may be returned subsequently to the stock bottle and used until exhausted, as shown by the precipitation of manganese dioxide. Any manganese dioxide stain is removed with ease by treating it with dilute sulphuric acid containing a little hydrogen peroxide.

PROCEDURE WITH BATHOPHENANTHROLINE SULPHONATE Wash two optical cells (1 cm. light path) with soap solution and a small plug of cotton wool wound round the end of a thin wooden stick. Rinse thoroughly, drain, and inspect the cells for freedom from visible organic matter; then fill them with the acid cleaning mixture. Return the acid to its container and thoroughly wash the cells, rinsing finally with ion-free water. Drain on to filter paper and wipe the outsides with soft tissue.

Into cell B (for blank) measure 1·75 ml. water and into cell T (for test) 0·5 ml. serum. To each add 0·75 ml. heated dithionite reagent. Mix the contents of cell T by gentle but thorough oscillation of the forefinger against the lower side of the cell. After four minutes add 1·25 ml. water to cell T and mix the contents of both B and T by up-and-down stirring with a thin glass rod flattened at the bottom; avoid the optical faces of the cells. Alternatively, mix with a Pasteur pipette. Read T against B in a spectrophotometer at 534 mµ, the wavelength of maximum absorption of the iron-(bathophenanthrolene sulphonate)$_3$ complex. Let the reading be A_1 units (linear absorbance or meter scale divisions). To T and B add 0·08 ml. bathophenanthroline sulphonate reagent by means of a 0·2 ml. pipette and mix. After two minutes read again in the instrument; let the reading be A_2 units. To T only add 0·04 ml. (1 µg.) standard iron 25 µg./ml. by means of a 0·1 ml. pipette. Mix and after one minute again read T against B. Let the third reading be A_3 units. Throughout the procedure it is best not to remove the cells from their holder in order to avoid errors. If a cell should be removed, replace it with the same optical face towards the detector of the instrument. When the measurements are carried out as described, the absorbance readings are relative to one cell only and accordingly the results are not affected by differences in cell characteristics. Consequently it is not absolutely necessary to use high quality optical glassware; in fact it should be possible to use any optically clear container such as a test tube, although this has not been investigated in the present work.

PROCEDURE WITH *O*-PHENANTHROLINE The procedure is much the same as before, the following volumes being used at each successive step:

(*a*) 1 ml. water in cell B; 1 ml. serum in cell T

(*b*) 1·5 ml. heated dithionite 3% reagent in each

(*c*) 0·08 ml. *o*-phenanthroline reagent in each
(*d*) 0·04 ml. (2 μg.) standard iron, 50 μg./ml. in T only

Readings in this case are made at 510 mμ, the wavelength of maximum absorption of the iron-(*o*-phenanthroline)$_3$ complex.

CALCULATION This is the same for both colour reagents. Ignoring volume changes:
Increment in linear absorbance on addition of standard iron $= A_2 - A_1$
Increment due to liberated serum iron $= A_3 - A_1$

$$\text{So that } \mu\text{g. iron in 1 ml. serum} = \frac{A_3 - A_1}{A_2 - A_1} \times 2$$

$$\text{and so, serum iron} = \frac{A_3 - A_1}{A_2 - A_1} \times 200\ \mu\text{g.}\%$$

If allowance is made for dilution factors to compensate for volume changes, the exact calculation is:

$$\text{Transferrin iron} = \frac{1{\cdot}03\ A_3 - A_1}{1{\cdot}05\ A_2 - 1{\cdot}03\ A_3} \times 200\ \mu\text{g.}\%$$

However, the error in using the approximate formula is usually less than 2 μg. % Fe and may be ignored for most purposes. For low values the more exact equation is preferred.

METHOD II: DETERMINATION OF LATENT IRON-BINDING CAPACITY

The preparative steps are the same as for serum iron determination.

REAGENTS These are as for the previous method, with one addition:
Normal saline-ascorbic acid Dissolve a knife-point (N 10 mg.) of ascorbic acid in 50 ml. normal saline. It is essential that the reagent be made under iron-free conditions, *i.e.*, using clean glassware, pure sodium chloride, and de-ionized water. No colour should be visible on addition of bathophenanthroline or its sulphonate.

PROCEDURE WITH BATHOPHENANTHROLINE Into cell B measure 0·58 ml. de-ionized water and 2 ml. saline-ascorbic acid reagent, and into T measure 0·5 ml. serum and 2 ml. saline-ascorbic acid. To T only, add 0·08 ml. (2 μg.) standard iron 25 μg./ml. and mix the contents of both cells. After two minutes read as before at 534 mμ. Let the reading be A_4 units. Within three minutes from the time of adding the iron, add to both B and T 0·08 ml. bathophenanthroline reagent and, after mixing, allow the solutions to stand for six minutes. Read again and check after further two-minute intervals. No further increase is usually observed after eight to 10 minutes. Let this reading be A_5 units. Finally, add 0·04 ml. (1μg.) standard iron, 25 μg./ml., to T only; mix, and after one minute read as before. Let this final reading be A_6.

PROCEDURE WITH *O*-PHENANTHROLINE If this reagent is used the volumes and procedures are modified as follows:
(*a*) To B add 1·08 ml. water and 1·5 ml. saline-ascorbic acid; and to T add 1 ml. serum and 1·5 ml. saline-ascorbic acid; mix.
(*b*) To T only add 0·08 ml. (4μg.) standard iron, 50 μg./ml. and proceed as before.
(*c*) Add 0·08 ml. *o*-phenanthroline reagent to both cells, and in this case allow the solutions to stand for 10 to 20 minutes until a constant reading is achieved.
(*d*) To T add 0·04 ml. (2 μg.) standard iron, 50 μg./ml., mix, and proceed as before.

All readings are made at 510 mμ for this reagent.

CALCULATION This is the same for either colour reagent and is carried out as follows:
Calculated absorbance from added iron if none were bound to transferrin $= 2\ (A_6 - A_5)$
Measured absorbance of residual iron complex $= A_5 - A_4$
Therefore absorbance of complex which would have been produced by the amount of added iron bound by transferrin
$= 2(A_6 - A_5) - (A_5 - A_4)$
Iron taken up by transferrin in 1 ml. serum

$$= \frac{2(A_6 - A_5) - A_5 - A_4)}{A_6 - A_5} \times 2\ \mu\text{g.}$$

Latent iron-binding capacity

$$= \frac{2(A_6 - A_5) - (A_5 - A_4)}{A_6 - A_5} \times 200\ \mu\text{g.}\%$$

As before, the exact expression is: Latent iron-binding capacity

$$= \frac{2(1{\cdot}05\ A_6 - 1{\cdot}03\ A_5) - (1{\cdot}03\ A_5 - A_4)}{1{\cdot}05\ A_6 - 1{\cdot}03\ A_5} \times 200\ \mu\text{g.}\%$$

but again the error involved in the use of observed linear absorbances is usually small, except for low values of the latent iron-binding capacity when the last expression should be used. It can be shown also that the error in the approximate expression may be larger for clear sera than for those with a higher background absorption.

The total iron-binding capacity is then the sum of the unsaturated transferrin iron level (TFe_u) and the latent iron-binding capacity.

Total iron-binding capacity $= TFe_u +$ latent iron-binding capacity, and the percentage saturation is given by:

$$\text{Saturation} = \frac{TFe_u}{\text{Total iron-binding capacity}} \times 100\%$$

EXPERIMENTAL RESULTS

RECOVERY EXPERIMENTS A few general remarks are necessary concerning the nature of the iron which is being determined. For analytical purposes the iron in natural serum may be considered to consist of (*a*) transferrin iron, extractable with heated dithionite; (*b*) non-specific iron, also extractable; and (*c*) other iron, not extractable with dithionite reagent. Hence method I will determine (*a*) and (*b*) only.

When iron is added incrementally to serum, the following effects are observed. Amounts less than those required to saturate the transferrin are taken up virtually quantitatively by the transferrin, and no significant increase in absorbance at 534 mμ is observed when bathophenanthroline sulphonate is

added to such sera after dilution with saline-ascorbic acid, indicating no non-specific uptake.

When amounts of iron more than those necessary to saturate the transferrin component are added, some of the excess iron is non-specifically absorbed, as evidenced by a rise in linear absorbance at 534 mμ when bathophenanthroline sulphonate is added to the diluted sera. This indicates that while bathophenanthroline sulphonate in saline-ascorbic acid has no effect on transferrin-bound iron it is a stronger complexing agent than non-specific centres in the serum and, under our conditions at least, fairly rapidly chelates all such iron. Further, if excess iron is added to serum and non-specific uptake allowed to proceed for 10 minutes, then all the added iron is recoverable, *i.e.*, is dithionite extractable. The conclusion from these observations is that the iron measured by methods I and II is of types (*a*) and (*b*). Further, natural serum contains no iron of type (*b*). Since analyses for total iron by a wet-ashing procedure have not been carried out in the present work, the magnitude of (*c*) remains unknown. Methods employing strong acid extraction might be expected to give results nearer to those obtained by wet-ashing procedures and, if type (*c*) iron is present, higher than those obtained by our method I. The results of various workers are compared later (see under Normal Values). As regards the values found by method II, the above observations and the recovery experiments show that this method finally measures only iron taken up by the unsaturated transferrin component of the serum, that is, type (*a*) iron.

Recovery experiments were carried out in order to test the potentiality of both methods. Having determined the serum iron and latent iron-binding capacity values of a number of sera, amounts of iron both less than and more than those required to saturate the transferrin were added to the sera, which were then analysed as before. Table I presents some typical results obtained by method I for four sera which were undersaturated; the fifth was fully saturated as regards the transferrin component and partially saturated as regards the non-specific components. In all cases, quantitative recovery of the added iron was obtained, whether this was of type (*a*) or of types (*a*) and (*b*) together. None of the added iron is converted to the irrecoverable type (*c*).

TABLE I

RECOVERIES OF IRON ADDED TO SERA USING METHOD I

Sample No.	*Fe (μg. %)*	*Added Fe (μg. %)*	*Recovered Fe (μg. %)*	*Percentage Recovery*
1[1]	27	0		
	121	100	94	94
	149	125	122	98
	177	150	150	100
2	39	0		
	139	100	100	100
	182	150	143	95
3	84	0		
	191	100	107	107
4	62	0		
	155	100	93	93
5[2]	32	0		
	138	100	106	106
	234	200	202	101

Mean recovery = 99·3 ± 7%

[1] *o*-phenanthroline reagent was used for samples 1 to 4.
[2] This serum had a latent iron-binding capacity of 97μg. %; bathophenanthroline reagent was used.

Similar 'recoveries' were carried out by method II. In this case the decrease in the latent iron-binding capacity was measured after undersaturating with 100 μg. % Fe. A typical experiment is shown in Table II. The latent iron-binding capacity was determined in duplicate on the original serum and in triplicate on the serum with added iron.

Such experiments showed that the uptake of iron by transferrin and the recovery of residual ionic iron are virtually quantitative under the conditions described.

TABLE II

TYPICAL 'RECOVERY' EXPERIMENT BY METHOD II[1]

Serum	*Absorbance Readings (A)*									*Latent Iron-binding Capacity (μg. %)*	*Mean (μg. %*
	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9		
Original	0·156	0·304	0·450	0·154	0·302	0·448				197·3	197·3
	Δ0·148	0·146		Δ0·148	0·146					197·3	
Plus 100 μg. % Fe	0·157	0·374	0·519	0·161	0·377	0·522	0·156	0·376	0·520	100·7	
										102·1	99·8
	Δ0·217	0·145		Δ0·216	0·145		Δ0·220	0·144		96·6	

Difference between means = 97·5 μg. %; expected difference = 100 μg. %
'Recovery' = 97·5%

[1] Bathophenanthroline reagent was used for these experiments.

TABLE III

REPRODUCIBILITY OF RESULTS FOR SERUM IRON AND LATENT IRON-BINDING CAPACITY

Serum No.	Colour Reagent	Serum Iron (μg. %)				Latent Iron-binding Capacity (μg. %)		
					Mean			Mean
1	Sulphonated bathophenanthroline	31·5	31·5	34·2	32·3	240·5	240·3	240·4
	o-phenanthroline	33·3	32·0		32·7	240·6	239·4	240·0
2	o-phenanthroline	52·6	56·4	57·7	55·6			
3	o-phenanthroline	83·3	84·6	84·6	84·2			

REPRODUCIBILITY Table III summarizes some typical results obtained from concurrent triplicate or duplicate analyses of the same serum. The reproducibility is reasonably satisfactory for either colour reagent; furthermore, the results obtained when the same serum is analysed with each reagent in turn are normally in good accord.

The standard deviation from the mean for serum iron values is ±3·3% so that the extreme confidence limits may be set at ±10% (Henry and Segalove, 1952).

The variations in latent iron-binding values for duplicate or triplicate determinations lie within ±3% of the mean as compared with a variation from the mean of ±6% for serum iron duplicates or triplicates, so that for determinations of the latent iron-binding capacity the extreme confidence limits of variation may be set at about ±5% of the mean value.

In both methods I and II the increases in absorbance on addition of standard iron in the last step of the determinations show little variation, so much so, that any marked deviation from the mean increment may be usually regarded as due to deterioration of the iron standards or to a pipetting error. The values appear to be little affected by the particular serum used. For a fresh standard we find that the increment does not vary by more than 0·003 linear absorbance units under the best conditions.

EFFECTS OF HAEMOLYSIS AND TURBIDITY It has been stated by some authors (*e.g.*, Webster, 1960) that dithionite will release iron from haemoglobin and falsify any measurement of serum iron. This point was investigated. A blood sample containing 13·2 g./100 ml. haemoglobin was diluted 1 : 100 with water and centrifuged. Varying amounts of the supernatant were added to a serum and the serum iron determined by method I. Identical results were obtained up to a level of about 0·3% haemolysis indicating that no haemoglobin iron is released under our conditions. Beyond this level precipitation occurred and rendered the results erratic. Sera which are obviously haemolysed should therefore be rejected as unsuitable for analysis. Similar results were obtained for determinations of latent iron-binding capacity.

It was found that slightly turbid sera could be analysed satisfactorily, providing the finally observed linear absorbances A_3 and A_6 fell below 0·8 units when using the bathophenanthroline reagent for both methods I and II. Above this reading, erratic results were obtained when a clear serum was compared with the same serum rendered turbid by addition of varying amounts of cholesterol in olive oil. In practice, moderate degrees of turbidity can thus be tolerated, but the methods will not deal satisfactorily with markedly lipaemic sera. A possibly more accurate procedure for method II is to replace the aqueous blank with a serum blank, when turbidity effects should cancel, providing the cells B and T are closely matched and of good optical quality.

EFFECTS OF AGEING AND TEMPERATURE ON SERUM A number of sera were analysed by methods I and II over periods ranging up to 140 hours from the time of taking the blood specimens. No appreciable change in values occurred for sera up to 24 hours' old which were left at room temperature. For sera refrigerated at 5° to 8° variable results were obtained, but in general somewhat lower values were found for both iron levels and latent iron-binding capacities. As the sera aged further, generally lower values were obtained and the results of triplicate analyses for serum iron became more and more erratic with refrigerated specimens. After 140 hours marked decreases and eccentricity in values for latent iron-binding capacity were observed, these being even greater with refrigerated sera. No advantage was found in warming refrigerated specimens to 37°C for 30 minutes. It is recommended therefore that specimens should be examined as soon as possible, in any case within 30 hours, refrigeration being contraindicated.

VARIATIONS IN THE NATURE OF THE EXTRACTOR IN METHOD I Sodium thiosulphate, potassium metabisulphite, hydrazine hydrate, sodium sulphite, sodium thiolacetate, and sodium dithionite were investigated at various concentrations in order to assess their powers of extracting and concomitantly reducing the transferrin iron in both fresh and aged serum. Sodium dithionite was much superior to the others as regards speed and efficiency of extraction and was selected as the most suitable reagent. It was then found that even fresh solutions of sodium dithionite in stabilizing acetate were incapable of extracting all the transferrin iron from sera which had aged for 24 hours or more. However, if the fresh dithionite-acetate solution was heated for five minutes in boiling water, the resultant solution was fully capable of extracting the iron from samples up to 30 hours old but not beyond that age. The nature of the extractor is not known. Sodium dithionite solutions are said to break down to a mixture of thiosulphate and metabisulphite in the absence of oxygen, or to sodium metabisulphite in the presence of oxygen (Partington, 1937). However, a mixture of equal volumes of sodium metabisulphite 6% and sodium thiosulphate 8% failed to extract iron completely within a period of 10 minutes. The mixture on heating behaved in a similar fashion to dithionite in that it turned yellow at 100° and became colourless on cooling to room temperature; but once again, the mixture failed to yield a full recovery of the serum iron. Likewise, a heated mixture of solutions of sodium metabisulphite and yellow ammonium sulphide, adjusted to *p*H 4 with acetic acid, extracted but little iron. Although sodium dithionite is not the ideal compound to use, as it is frequently impure and contains variable amounts of iron, no suitable alternative could be discovered. The material employed (B.D.H. laboratory reagent grade) was fairly satisfactory as regards iron content and gave acceptably low blanks (corresponding to about 0·1 μg. Fe per 0·75 ml. reagent).

Ascorbic acid in low concentration appears to have no effect on bound transferrin iron, so that in method II it is an ideal compound for the reduction of free ionic iron without disturbing the binding of transferrin iron.

NORMAL VALUES Sera from 12 males and 14 females were examined by methods I and II using *o*-phenanthroline as colour reagent. The subjects were active blood donors with normal haemoglobin levels. The number of donations given previously varied from 0 to 41 and these were usually made at three-monthly intervals. If any subject who had made more than six previous donations had a serum iron level or a latent iron-binding capacity outside the normal ranges found by previous workers, these values were rejected. Data from analyses of the sera of new donors were included, whatever their values. The findings are presented in Table IV. The ages of the donors ranged from 18 to 60 years. No attempt was made to investigate any possibility of diurnal variation in serum iron levels, all specimens having been collected between 9 and 11.30 a.m. from non-fasting donors. (No serum had to be rejected because of too great a turbidity.) Neither was any investigation made of possible variations in levels in female subjects at various stages of the menstrual cycle.

Compared with the results of previous workers (see Davies, Levin, and Oberholzer, 1952, for a

TABLE IV

NORMAL VALUES FOR SERUM TRANSFERRIN IRON AND IRON-BINDING CAPACITY

Males

No.	*Age*	*Previous Donations*	*Serum Fe (μg. %)*	*Latent Iron-binding Capacity (μg. %)*	*Total Iron-binding Capacity (μg. %)*	*% Saturation*
1	43	0	140	156	296	47
2	23	3	78	199	277	28
3	22	0	106	165	271	39
4	38	1?	150	303	453	33
5	32	1	83	195	278	30
6	38	20	76	174	250	30
7	32	30	114	200	314	36
8	24	1?	104	155	259	40
9	20	5	147	104	251	59
10	58	41	160	115	275	58
11	32	4	63	151	214	29
12	37	0	84	151	235	36
Means			109	172	281	39
Ranges			63-160	104-303	214-453	28-59

Females

No.	*Age*	*Previous Donations*	*Serum Fe (μg. %)*	*Latent Iron-binding Capacity (μg. %)*	*Total Iron-binding Capacity (μg. %)*	*% Saturation*
1	19	2	88	183	271	32
2	44	0	92	146	238	39
3	21	0	142	209	351	41
4	21	5	77	259	336	23
5	31	0	27	175	202	13
6	39	4	94	127	221	43
7	20	5	109	116	225	48
8	18	0	39	158	197	20
9	60	17	84	164	248	34
10	34	3	50	217	267	19
11	23	0	96	136	232	41
12	49	1	92	180	272	34
13	35	—	94	187	281	33
14	20	—	62	164	226	27
Means			82	173	255	32
Ranges			27-142	116-259	197-351	13-48

summary) our values for serum iron levels are somewhat lower in male subjects and decidedly lower for females. Since the values previously found have nearly all been based on precipitation methods with strong acid extraction procedures, it would appear that such methods come closer to determining total serum iron than the present ones, which probably give more nearly a measure of the iron bound to transferrin only. Our markedly lower mean value for females might indicate that a higher proportion of the serum iron is of type (*c*) than is the case for males. All workers, with the possible exception of Cartwright and Wintrobe (1949), find lower values for females than for males, the differences ranging from 12 to 27 μg. Fe % (including the present findings).

Again, as regards latent iron-binding capacity levels, our results fall below those previously obtained. With methods such as those of Rath and Finch (1949), the increments in linear absorbance are relatively small and errors of unknown magnitude may occur, particularly with sera containing any turbidity; little seems to have been published concerning confidence limits for this type of method so that comparisons with the present one are difficult to make. For females, the mean values by both methods are considerably lower than those of other authors. For males, however, other values are similar to those of Rath and Finch (1949).

In spite of the discrepancies between the normal values published for serum iron, and latent and total iron-binding capacity levels, the figures for percentage saturation found by all workers are in tolerably good accord, being $38 \pm 4\%$ for males and $33 \pm 1\%$ for females. A comparative study of the various methods is evidently needed.

DISCUSSION

The method described for serum iron determination is based on the series of reactions:

$$TFe_u \xrightarrow[Na_2S_2O_4]{\text{heated}} T + Fe^{2+} \xrightarrow[\text{phen}]{} Fe(phen)_3$$

The success of the method depends on two main requisites. First, the transferrin iron must be completely removed and reduced to the ferrous state; the recovery experiments indicated that such is the case. Secondly the background absorption due to turbidity and serum chromogens must remain stable throughout the procedure. This was checked with a number of sera by running the absorption spectra on a recording spectrophotometer at each stage of method I, beginning at wavelengths higher than those at which the iron complex absorbs. Coincidence of the spectra in this region (700 to 660 mμ) was usually observed, any deviations being of a minor order, little more than would be expected as the result of the dilution effect of added reagents. The reasonable assumption could then be made that the background absorption at 540 mμ likewise was unaltered to any extent. Again, substantially constant and quantitative recoveries of added iron would indicate that no serious fluctuations in background occur at any stage, so that the method may be used with confidence.

Method II, for the determination of the latent iron-binding capacity of serum, is based on the following scheme:

(a) $P + TFe_u + Fe^{2+}_A \longrightarrow TFe_{sat.} + PFe_p + Fe^{2+}_R$
$\downarrow$ phen $\downarrow$

(b) $PFe_p \longrightarrow P + Fe_p(phen)_3$
$\downarrow$ phen $\downarrow$

(c) $Fe^{2+}_R \longrightarrow Fe_R(phen)_3$

(d) $Fe_R(phen)_3 = Fe_R(phen)_3 + Fe_p(phen)_3$

where:

P = non-transferrin proteins or serum constituents
TFe_u = transferrin in original serum
$TFe_{sat.}$ = saturated transferrin component
Fe^{2+}_A = added iron
PFe_p = unsaturated non-specific iron complexes
$Fe_p(phen)_3$ = iron complex formed from non-specifically bound iron
Fe^{2+}_R = added ionic iron not bound to T or P
Fe_R = total residual added iron not bound to transferrin, after establishment of equilibrium

For the method to be successful, the following ideal conditions must be fulfilled as closely as possible: (1) As before, background should be unaltered; (2) the complexing agent should completely remove non-specifically bound iron (scheme, part (b)); (3) the complexing agent should not affect the transferrin-bound iron; (4) the reducing agent used to keep ionic iron in the ferrous state should not liberate transferrin-bound iron.

Condition (1) was investigated spectrophotometrically as for method I. Small departures from background constancy were noted in some cases, somewhat larger than for method I, but no way could be found of allowing successfully for these changes apart from including a serum blank. For the majority of sera, however, variations are small and consistent for any one serum as judged by the high reproducibility obtainable with replicate determinations. For larger variations possible errors are difficult to

estimate, since any change in background absorbance at 540 mμ remains occult.

Conditions (2), (3), and (4) have been discussed already and appear to be closely obeyed when the procedure described is followed. For the majority of sera, therefore, it appears that method II gives a reasonably reliable determination of the latent iron-binding capacity.

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Lipids in cadaver sera after fatal heart attacks

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SYNOPSIS Blood lipids have been measured in sera from 750 cadavers. All nine fractions measured show a remarkably consistent pattern of differences between the various gross morbid anatomical forms of ischaemic heart disease. The highest mean values are found when an obvious coronary thrombus is present and those in simple atheromatous occlusion are nearly as high. When there is recognizable necrosis the values are considerably more normal.

The serum lipids show relatively little change due to autolysis, about one half of 1 % per hour or less.

Lipoprotein levels assayed with *Cl.welchii* alpha toxin correlate well with disease states.

Despite the precision of modern clinical diagnosis details of many morbid anatomical states can still only be ascertained at necropsy. Thus, in ischaemic heart disease, myocardial necrosis can confidently be predicted in a clinical attack but the presence or nature of arterial obstruction can only be inferred. In consequence, in most biochemical investigations, the label coronary thrombosis is used as a synonym but, whatever the frequency of minor mural thrombi, gross thrombi occluding the lumen are in fact relatively uncommon. This report describes consistent differences in the serum lipids between various morbid anatomical variants of the syndrome in a series of fatal cases. It also describes the effect of autolysis on the lipids, and contains data on the elegant method of Perrin (1959) for assessing lipoprotein with *Cl. welchii* alpha toxin lecithinase.

MATERIAL AND METHODS

About 750 cadaver arm vein sera have been examined over four years (Enticknap, 1960a and b). About 200 were rejected because of infection or haemolysis. Most of the subjects had died unexpectedly and they are grouped by causes of death in Table I. Atherosclerosis was assessed in six sites giving an index up to a maximum of 24. No special techniques were used to discover small thrombi, the cases being diagnosed as coronary thrombosis or atheromatous occlusion depending on whether the lumen was occluded by ante-mortem clot obvious to the naked eye after multiple incisions.

TOTAL SERUM CHOLESTEROL This was estimated by a Liebermann-Burchard colour development technique using the rather large amount of 0·2 ml. sulphuric acid. The acid ferric chloride methods give very high blanks with cadaver sera.

TOTAL SERUM ESTERIFIED FATTY ACIDS The triolein standard of Stern and Shapiro (1953) was used. The 95% range of 104 normal blood donors was 5·7-12·3-18·9 mEq./l. and there was no sex difference.

LIPOPROTEIN Perrin's (1959) technique was simplified: the change in opacity in 18 hours at 37°C. of 0·3 ml. serum in 3 ml. Sorenson's borate buffer *p*H 7·61 containing 0·025 M $CaCl_2$ and 0·8 Wellcome units of *Cl. Welchii* alpha toxin (2 mg. of preparation AGX 1552) in a 1 cm. cup at 650 mμ was directly recorded. Electrophoresis allowed visual assessment of the fractions (Dangerfield and Smith, 1955) and prebeta lipid was recorded in Smith (1957) units. Beta-lipoprotein was also recorded by a modification of the original polysaccharide flocculation phenomenon of Bunstein and Samaille (1956); the opacity developed by 0·2 ml. serum in 2·0 ml. of 0·06 M $CaCl_2$ on treatment with 0·04 ml. 1% calcium heparin was referred to Kunkel's (1947) barium sulphate standard, and assigned a value of 10 units with an Ilford 609 filter. The 95% range of 104 normal blood donors was 3·0 - 9·6 - 16·1 units and the original data suggest that one unit is about 0·4 g.

RESULTS

There are slight changes in some of the lipid values with time elapsing between death and necropsy (Table II). They show a diphasic pattern of accumulation in the serum around a skew rising mean with a declivity at around the end of the second day. In disease groups which were large enough to give

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TABLE I

CLASSIFICATION BY CAUSES OF DEATH AND DISTRIBUTION OF SERUM LIPIDS

	Ischaemic Heart Disease					*Other Forms of Heart Disease*	*Other Forms of Arterial Disease*	*Control Cases*
	Coronary Occlusion by Atheroma	*Myocardial Fibrosis*	*Myocardial Necrosis*	*Coronary Thrombosis*	*All Cases*			
Average age in years	62	74	69	68	66	68	69	62
Proportion of males to females	1·7	0·8	0·9	1·1	1·2	1·9	0·5	0·9
Index of atherosclerosis	12	12	14	13	13	8	10	6
Total serum cholesterol								
Mean value (mg./100 ml.)	262	251	205	*306*	260	197	231	175
Standard deviation	83	65	78	85	83	78	90	71
Number of observations	17	15	15	7	54	11	14	46
Total serum fatty acids								
Mean value (mEq./l.)	*19·3*	13·7	15·1	15·5	15·7	11·9	15·9	12·9
Standard deviation	8·4	3·9	5·7	5·0	7·1	4·3	9·2	5·0
Number of observations	71	13	41	27	152	25	55	85
Total lipoprotein								
Mean values $\left(\text{alpha toxin } dE\,\frac{650}{1\text{cm.}}\right)$	0·53	0·45	0·35	*0·54*	0·47	0·28	0·45	0·30
Standard deviation	0·27	0·40	0·32	0·24	0·35	0·21	0·33	0·18
Number of observations	39	7	21	18	85	20	33	43
Beta lipoprotein								
Mean value (heparin units)	16·9	14·1	14·1	*22·7*	16·1	11·8	15·3	11·0
Standard deviation	6·0	4·5	6·9	2·5	6·5	7·0	7·5	5·7
Number of observations	12	7	8	3	30	8	7	35
Mean value (electrophoretic units)	1·4	1·0	1·4	*1·6*	1·4	1·0	1·0	1·1
Standard deviation	0·48	1·0	0·73	0·6	0·74	0·85	0·68	0·77
Number of observations	71	11	38	23	143	17	27	92
Mean value (Smith units of pre-beta lipid)	2·0	1·6	2·3	*3·1*	2·2	1·4	1·5	1·2
Standard deviation	1·61	1·82	1·64	1·94	2·24	1·58	1·41	1·55
Alpha/beta ratio								
Mean value (normal = 1·0)	0·41	0·54	0·65	*0·35*	0·47	0·81	0·64	0·67
Alpha lipoprotein								
Mean value in electrophoretic units	0·7	*0·5*	0·8	0·7	0·7	0·7	0·8	0·7
Mean opacity of serum diluted 1 : 10 in borate buffer $\left(E\,\frac{1\text{cm.}}{650}\right)$	*0·23*	0·19	0·19	0·18	0·21	0·20	0·19	0·16

TABLE II

CHANGES IN BLOOD LIPIDS WITH INCREASING INTERVALS BETWEEN DEATH AND NECROPSY

	0-12 Hours	*13-24 Hours*	*25-36 Hours*	*37-48 Hours*	*49-60 Hours*	*More than 60 Hours*	*Mean Hourly Increment*
Cholesterol (mg. per 100 ml.)							
Mean value for all sera tested	195	207	213	232	200	215	0·5
Total fatty acids (mEq./l.)	11·8	13·2	13·4	15·8	11·2	13·6	0·04
Beta lipoprotein							
(a) heparin units	8·3	11·3	13·5	8·5	15·0	14·9	0·1
(b) Sudan black units after electrophoresis	0·9	1·2	1·4	1·2	1·4	1·4	0·01
(c) Prebeta lipid in Smith units	1·1	1·8	2·0	1·8	2·1	2·1	0·01
Lipoprotein (Perrin) $\left(\frac{dE\ 1\text{cm.}}{650}\right)$	0·49	0·35	0·37	0·50	0·35	0·40	—
Alpha/beta lipoprotein ratio (normal = 1)	0·8	0·5	0·8	0·5	0·5	0·7	—
Alpha lipoprotein	0·6	0·7	0·8	0·7	0·8	0·7	—
Gamma globulin	1·2	1·2	1·3	1·2	1·2	1·3	—
Serum opacity	0·05	0·18	0·30	0·18	0·16	0·12	—

informative means the pattern was similar. A mean hourly increment was calculated from lines of best fit. The disease groups were all of comparable mean age and the necropsies were performed at fairly uniform mean time intervals.

The mean values of the serum lipids have been

grouped (Table I) to show the changes attributable to ischaemic heart disease, its four morbid anatomical variants of simple atheromatous coronary occlusion, myocardial fibrosis, myocardial necrosis, and coronary thrombosis, and to enable the effects of arterial disease not primarily affecting the heart (systemic atherosclerosis) and heart disease not due to vascular occlusion to be assessed. All other causes of death formed the control group, with the exception of cancer which caused uniformly low lipid values and so was excluded. This control group was analysed into six components but no consistent differences were seen.

There was a close correlation between the serum fatty acid content and lipoprotein measured by lecithinase with lipaemia assessed both with the naked eye and photometrically (Fig. 1). Eight out of 10 milky sera occurred in ischaemic heart disease. Numerous calculations show that haemolysis does not regularly affect the various lipid fractions of

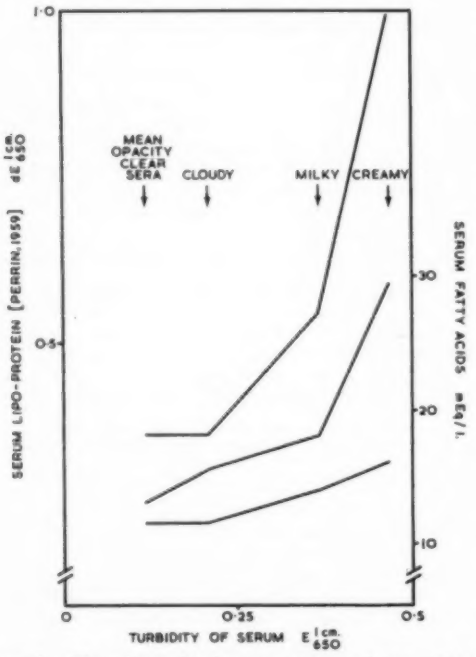

FIG. 1. *The relationship between serum turbidity and its content of lipoprotein and fatty acids. The four points represent groups of sera considered visually to be comparable. In the top line the mean opacity of groups of cadaver sera is plotted against lipoprotein content (Perrin, 1959), in the second line against the mean fatty acid content of these same groups, and in the third line against the mean fatty acid content of groups of blood donor sera.*

cadaver sera nor do they correlate with the index of atherosclerosis at all closely. There was no consistent relationship with the state of the stomach contents and, compared with the controls, there was no significantly greater frequency of recent ingestion in the subjects with ischaemic heart disease. There is a significant, but not close, correlation between the individual values of fatty acids and alpha-toxin units of lipoprotein in 200 sera ($r = 0{\cdot}33$; s.e. 0·071).

DISCUSSION

These four semantic variants of ischaemic heart disease are commonly used on death certificates despite the overlap between their morbid anatomical counterparts. Their proportions of approximately 43, 10, 25, and 15% respectively are quite characteristic of the more sudden seizures. This investigation was begun to elucidate those cases, here referred to as coronary occlusion, in which death was undoubtedly due to acute cardiac ischaemia but in which careful *post-mortem* dissection does not reveal any recent gross changes in myocardium or arteries. As they show no obvious thrombotic occlusion and, indeed, unless special techniques are used, no thrombi at all, the mechanism of death is obscure. Enzyme assays are consistent with the absence of necrosis in these cases (Enticknap, 1960b). This investigation shows abnormalities of their serum lipids which present rather more clear differences than those found when necrosis is established. Further, it demonstrates that while the most abnormal lipids are found in those cases with an obvious thrombus, the cases of occlusion resemble them fairly closely.

Autolytic changes must be considered. Laves (1960) has shown that the ultra-violet absorption spectrum of the blood is changing even within the moments before death. Other substances, particularly serum enzymes, also change with extreme rapidity (Enticknap, 1960b) and rise to very high levels. Serum lipids, on the other hand, although showing qualitatively similar changes, are quantitatively much less altered, and corrections based on the increments in Table II do not affect the argument. In 1936, Landé and Sperry, at Schonheimer's suggestion (Sperry, 1957), made *post-mortem* cholesterol analyses and Faber (1946) considered that any differences be encountered in serial estimations were within the limits of experimental error. More recently Paterson and Dyer (1959) have measured *ante-* and *post-mortem* cholesterol analyses in a closed population and concluded that they were comparable, provided that death was sudden, and Glanville (1960) has reported on hospital subjects. Schlang and Davis (1958), Schleyer (1954), and Spain, Bradess, and Greenblatt (1954) have all reported satisfactory

separations of cadaver serum proteins. It appears legitimate, therefore, directly to correlate lipid analyses on cadaver sera with disease states.

While the level of total esterified fatty acids appears to be the most useful test in confirming a necropsy diagnosis of simple coronary occlusion as a cause of death, the other fractions all appear to be of about equal value. Perrin's technique for total lipoprotein yields results with a fairly wide spread of differences between the syndromes and also gives very high values in the puzzling cases of occlusion, but the discrimination between the various groups is insufficiently good to justify the routine use of this rather complex method. He shows that it correlates with phospholipids in his Fig. 1 but no coefficient is given. In this series a loose relationship with total esterified fatty acids is shown but the technique clearly does not simply measure this parameter.

The lipids are most raised in the ischaemic heart disease group and moderately raised in the systemic atherosclerosis group, and many of the differences attain conventional significance ($p<0.05$). Thus ischaemic heart disease has a significantly higher mean cholesterol level than heart disease not due to vascular occlusion (standard error of difference 25 mg./100 ml.) and than the control group (s.e. diff: 15), but not than systemic atherosclerosis (s.e. diff: 27). Heart disease not due to vascular occlusion, on the other hand, closely resembles the controls, there being in fact no significant differences (s.e. diff; for pre-beta lipid: 0·34). Beta-lipoprotein and pre-beta lipid also differ significantly in the ischaemic heart disease and systemic atherosclerosis groups (s.e. diff: 0·147 and 0·103). Data from 13 of the cases of Landé and Sperry (1936) and from 28 of Faber's (1946) cases can also be arranged to compare these groups and show a similar small mean cholesterol difference to that reported here (31, 28, and 29 mg. per 100 ml. respectively).

The differences between the various forms of ischaemic heart disease are, however, more interesting and cannot be determined in clinical cases. The lipids in myocardial fibrosis, in which the heart failure is less sudden, are among the lowest recorded in fatal atherosclerosis and closely resemble those of non-cardiac arterial disease, there being no significant differences in this series. The mean fatty acid level is significantly lower than in coronary occlusion (s.e. diff: 1·66) and most of the other values differ markedly from those found when a thrombus is present.

Coronary thrombosis, on the other hand, is associated with the most abnormal pattern. Beta-lipoprotein is significantly greater than in any other group (s.e. diff. from coronary occlusion; 2·25) and so is pre-beta lipid (s.e. diff. from myocardial necrosis 0·22). This is in fact the biggest mean increase of any fraction, being more than 2·5 times that of the control group mean. The mean cholesterol level (s.e. difference 38), total lipoprotein (s.e. diff. 0·04), and the alpha-beta ratio all differ significantly from those of myocardial necrosis although not from those of the rest of the group. Thus the cases of myocardial necrosis have a significantly lower cholesterol, total lipoprotein, beta lipoprotein, and pre-beta lipid, and higher alpha-beta ratio than cases in which a thrombus was present.

The cases of coronary occlusion by uncomplicated atheroma share the highest mean levels with the thrombotic cases. Significant differences are seen only in beta lipoprotein and in pre-beta lipid but the serum fatty acids are higher in this class than in any other. Apart from this all the other most abnormally raised mean values occurred in the group of cases of coronary thrombosis (italics in Table I).

Two conclusions emerge. First, when cases of fatal ischaemic heart disease are grouped by conventional naked-eye necropsy criteria consistent differences are observed in the levels of the serum lipids. This fact is consistent with the hypothesis that the causes of death certified may have different mechanisms. Secondly, stoppage of the heart when the myocardium is apparently normal or when necrosis is minimal occurs when the blood lipids are most markedly abnormal. The more abnormal they are the more likely is a large thrombus to be present; established necrosis, on the other hand, is found when the lipids are much less abnormal. In any event the similarity between the findings in simple and thrombotic occlusion is confirmatory evidence that the former may indeed cause death when there is no evidence of gross recent thrombotic or necrotic change.

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Serum alkaline phosphatase fractionation as an aid to diagnosis

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SYNOPSIS A case is reported in which the serum alkaline phosphatase reached high levels, and in which the differential diagnosis between hepatic secondary deposits and osteomalacia proved difficult by the usual means. Separation of the alkaline phosphatases by starch grain electrophoresis was used as an aid to differential diagnosis.

The positive identification of secondary deposits in the liver by other than clinical means is sometimes difficult. Recent work by Keiding (1959) indicates that the serum alkaline phosphatase activity can be separated electrophoretically into components of hepatic and of bony origin, a technique which might be used in those cases presenting with a greatly raised level of enzyme of doubtful origin. Cases in which the diagnosis cannot be made on careful assessment of clinical, radiological, and routine biochemical evidence will be rare, but we present such a problem which clearly illustrates the validity and utility of the phosphatase fractionation.

HISTORY AND CLINICAL EXAMINATION

M.O., aged 73, was admitted to the Westminster Hospital under Mr. F. d'Abreu on 8 September 1960. In 1951, she had had a partial gastrectomy for a chronic, benign gastric ulcer, after which she developed diarrhoea, and this had continued with such severity that she had up to seven motions a day. In 1958, she had an annular growth at the rectosigmoid junction resected. This was shown histologically to be a grade II adenocarcinoma penetrating muscle (Dr. A. Morgan). During laparotomy the liver was examined and found to contain no palpable secondary deposits. In the week preceding operation the mean 24-hour faecal fat excretion on three specimens was 7·4 g. The serum potassium level was 3·2 mEq./l. and total protein 5·7 g./100 ml. On this evidence she seemed to have some malabsorption. On the present admission she complained that she had become worse and that she had lost half a stone in three weeks. She ate little, and this, combined with the malabsorption, could have accounted for her symptoms. The liver, spleen, and kidneys were not palpable, and there was no obvious lymphadenopathy.

INVESTIGATIONS She was anaemic with a haemoglobin of 54% (7·4 g./100 ml.), the erythrocytes showed anisocytosis and poikylocytosis. The total leucocyte count was 11,400/c.mm., of which 93% were polymorphonuclears, with an occasional myelocyte, and a 'shift to the left'. Platelets appeared normal. Bone marrow aspiration showed a toxic marrow. There was an increase of nucleated red cells but no megaloblasts. No malignant cells were seen. Radiologically the bones showed no evidence of metastases.

The serum potassium level was now 2·8 mEq./l. calcium 8·0 mg./100 ml., and phosphate 2·8 mg./100 ml. The serum alkaline phosphatase level was markedly raised to 87 K.-A. units, the pseudocholinesterase level was 32 units (normal 55-100 units), and lactic dehydrogenase 520 and 540 units on two occasions (normal 0-500 units). The urine contained a trace of urobilinogen, but other routine liver function tests were normal. Electrophoresis of serum showed a low albumin level and relatively raised α_1 and γ globulin levels. The total serum protein level was 5·20 g./100 ml., and albumin 2·93 g./100 ml.

TREATMENT AND PROGRESS In the absence of definitive evidence of metastases the patient was treated as a case of malabsorption with large doses of calciferol, iron, and folic acid, and also with plasma and blood transfusions. There was temporary clinical improvement during which the alkaline phosphatase level, which had risen to 96 K.-A. units, fell steadily to 76 units, and the serum Ca and PO_4 rose to 9·0 mg./100 ml. and 5·0 mg./100 ml. respectively on 24 November 1960. The serum protein and haemoglobin levels rose only temporarily following transfusion, and the clinical improvement was short-lived and she died on 1 December.

FRACTIONATION OF ALKALINE PHOSPHATASE

METHODS Serum, 2·5 ml., was dialysed overnight against borate buffer (*p*H 8·3, $\mu = 0{\cdot}075$) containing 12·3 g./l tre $MgSO_4 \cdot 7H_2O$, and then fractionated by starch block electrophoresis (Kunkel, 1954) in a tray 25 cm. × 5 cm ×

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1 cm. After running for six hours at 500 volts (during which time the temperature was maintained below 10°C.) the slab was sectioned at 1 cm. intervals and each segment extracted with two lots of 2 ml. of 0·9% NaCl to which had been added 12·3 g./litre $MgSO_4 7H_2O$. Protein content was estimated in each tube spectrophotometrically at 210 μ (Tombs, Souter, and Maclagan, 1959), using a 1 in 10 dilution with saline. Alkaline phosphatase was estimated on the Auto Analyzer (Technicon Instrument Co. Ltd.) using a modification of the King-Armstrong method (unpublished work). Finally paper electrophoresis was carried out on those fractions showing alkaline phosphatase activity.

RESULTS Alkaline phosphatase fractionation were carried out before and after treatment with calciferol. Figure 1 shows the electrophoretic protein and alkaline phosphatase patterns obtained with two sera, before and after treatment with calciferol, recovery of alkaline phosphatase being approximately 50%.

The fall in total activity from 96 to 76 K.-A. units between the first and second fractionations was entirely due to depression of β globulin activity. The α_1 peak remained essentially constant while the α_2 peak increased.

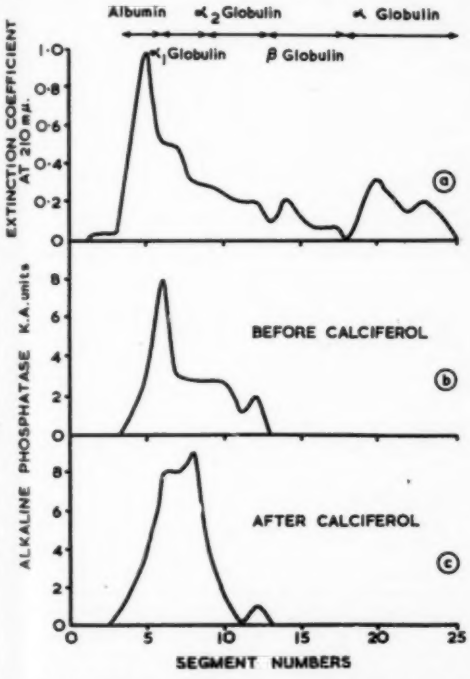

FIG 1. *(a) The protein pattern (b) The alkaline phosphatase pattern before treatment with calciferol. The phosphatase activity in segments 11 to 13 is that usually referred to as β phosphatase (c) The alkaline phosphatase pattern after treatment with calciferol.*

POST-MORTEM FINDINGS

Dr. D. H. MacKenzie found that the liver was slightly enlarged with almost the whole of the right lobe replaced by solid tumour tissue. The left lobe showed multiple small metastases. There was no evidence of bony metastases.

DISCUSSION

It was thought unlikely that a serum alkaline phosphatase level of 96 K.-A. units was due solely to osteomalacia and that a possible explanation was the presence of hepatic metastases from the rectal carcinoma. Although there was no clinical, radiological, or haematological evidence for this, the small urinary urobilinogen excess and the abnormal serum enzyme values supported the hypothesis but further evidence seemed desirable.

Keiding (1959) has shown that serum alkaline phosphatase can be separated into three components by starch grain electrophoresis. Of these, the β globulin activity is associated with phosphatases arising from bone whereas the α_1 and α_2 globulin activities are associated with liver phosphatase. His results indicate that this method might prove of value in detecting liver metastases. Rosenberg (1959), using a similar technique, failed to resolve the α_2 and β phosphatases and found that in a series of 60 cases the α_1 fraction never contributed more than 16% of the total activity. Our case showed some 50% of the total activity in the α_1 peak, a finding confirmed by paper electrophoresis of the active fractions, but the β globulin activity was never grossly raised.

The evidence of the phosphatase fractionations was that the initially high value was due to enzyme travelling with α_1 and α_2 globulins and was, therefore, mainly of hepatic origin. Although a grossly abnormal $\alpha_1 : \alpha_2$ phosphatase ratio was observed we feel that we have insufficient experience with other conditions to draw any conclusions from this finding. The fall after calciferol therapy was entirely in the fraction originating from bone (β globulin), suggesting that this also had been slightly raised at first. It appeared, therefore, that there was osteomalacia but no bony metastases, and that the enzyme came predominantly from liver deposits. This was amply confirmed by the very temporary response to therapy and by the necropsy findings.

We thank Mr. F. d'Abreu for permission to study this case, Professor N. F. Maclagan for his help, Mr. B. Allen for technical assistance, and Dr. J. Humble for allowing us to report the haematological findings.

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The determination of 3-methoxy 4-hydroxy mandelic acid in urine

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SYNOPSIS A simple and accurate method for determining 3-methoxy 4-hydroxy mandelic acid in urine is described. The amounts excreted in urine in 24 hours by nine normal subjects and three patients with phaeochromocytoma have been determined.

The determination of 3-methoxy 4-hydroxy mandelic acid (vanillyl-mandelic acid) in urine is of importance in detecting the increased production of this major metabolite of adrenaline and noradrenaline in patients with phaeochromocytoma (Gitlow, Khassis, Cohen, and Mendlowitz, 1959) and also in investigations on hypertension in which there may be disturbance of catecholamine metabolism leading to increased excretion of vanillyl-mandelic acid (Studnitz, 1960). Various methods for estimating it in urine have been described (Armstrong, McMillan and Shaw, 1957; Studnitz and Hanson, 1959; Sandler and Ruthven, 1959; Gitlow *et al.*, 1959; Robinson, Ratcliffe, and Smith, 1959), but none lends itself to rapid routine investigations with large numbers of urine samples. The method to be described is suitable for this purpose and can be carried out without recourse to special techniques such as paper chromatography, column chromatography, or high voltage electrophoresis. It is based on the fact that the azo dyestuff formed when vanillyl-mandelic acid is coupled with diazotized p-nitroaniline can be separated from interfering coloured substances by extraction from potassium carbonate solution into chloroform from which it can be re-extracted with sodium hydroxide to give a red solution suitable for colorimetry. In developing the method satisfactory conditions for selectively and quantitatively extracting vanillyl-mandelic acid from urine have been determined and the reaction between it and diazotized p-nitroaniline has also been studied in order to obtain maximum reproducibility.

PROCEDURE

REAGENTS In addition to ether A.R., chloroform B.P., and sodium chloride A.R. the following are made up:—

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p-Nitroaniline reagent To 0·5 g. p-nitroaniline add 3 ml. concentrated HCl and make up to 500 ml. with distilled water, any undissolved solid being filtered off. This solution stores indefinitely. To prepare the reagent add 1 volume of 0·05% (w/v) sodium nitrite A.R. to 1 volume of p-nitroaniline solution, mix and leave two min. then add 1 volume of 0·025% (w/v) ammonium sulphamate to destroy traces of nitrous acid. Prepare the nitrite and sulphamate just before use. The reagent can be used for up to two hours after preparation.

Potassium carbonate solution A 10% (w/v) solution of potassium carbonate A.R. in distilled water.

Standard vanillyl-mandelic acid solution Dissolve 10 mg. DL-3 methoxy-4 hydroxy mandelic acid (Californian Corporation for Biochemical Research) in 100 ml. of distilled water. The solution can be stored at −20° for at least six months and can be kept safely for several weeks at 4°. Standards are prepared as required by appropriate dilution with distilled water.

Magnesium oxide (for chromatography) Mix 1 part by weight of light magnesium oxide (May and Baker L 354) with 3 parts of cellulose powder (Whatman standard grade), any small lumps of the oxide being broken up in a pestle and mortar or small mill before mixing with the cellulose.

Methanolic KOH Dissolve 0·1 g. KOH A.R. in 100 ml. methyl alcohol.

Sodium hydroxide N/10

METHOD

To 2 ml. of urine add 3 ml. of distilled water, 0·5 ml. of concentrated HCl and sufficient solid sodium chloride to saturate the solution and give a slight excess. Extract with five successive 40 ml. volumes of ether, shaking well at each stage. Remove the extracted vanillyl-mandelic acid from ether into aqueous solution by shaking the combined ether extracts with 25 ml. of distilled water containing sufficient N/10 NaOH (about 1 ml.) to give a neutral extract followed by 15 and 10 ml. portions of distilled water. Develop colour by adding to the combined aqueous extracts (which should be neutral to

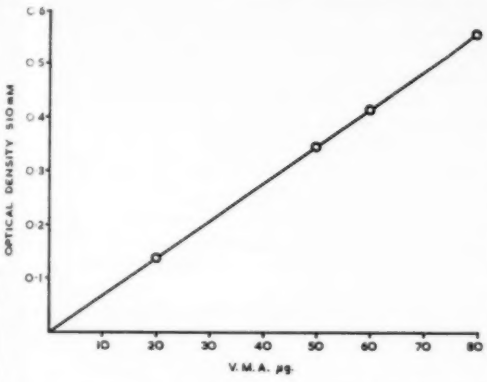

FIG. 1. *Curve relating optical density of diazo-derivative of vanillyl-mandelic acid at 510 mμ to concentration of vanillyl-mandelic acid.*

indicator paper) 5 ml. of p-nitroaniline reagent, leave for two min., then add 5 ml. of 10% potassium carbonate solution and leave a further five min. to allow maximum colour to develop. Remove the vanillyl-mandelic acid-diazo compound from the strongly coloured reaction mixture by shaking with successive 15, 10, and 5 ml. volumes of chloroform. The clear yellow chloroform solution obtained contains the un-ionized form of the complex. Extract into aqueous solution with 10, 5, 3, and 2 ml. of N/10 NaOH, adjust the volume of the combined extracts to 20 ml., and read the resulting red solution in a spectrophotometer at 510 mμ. A small amount of coloured impurity is left behind in the chloroform layer after extraction with NaOH. The spectrophotometer reading is converted into weight of vanillyl-mandelic acid by reference to a standard curve prepared with a solution of known vanillyl-mandelic acid using the same conditions for colour development as those used for the urine sample but omitting the ether extraction stage. A standard curve is shown in Fig. 1. The spectrophotometer used was a Unicam SP 600 using 1 cm. cells.

The coupling reaction is very sensitive to light and it is therefore necessary to work out of direct sunlight and in subdued daylight or preferably artificial light to avoid losses and erratic recoveries. The chloroform extract of the vanillyl-mandelic acid complex is also unstable in the presence of light and the extraction with sodium hydroxide should therefore be carried out with the minimum of delay. The final red colour is stable for at least 24 hours.

Normally the separation of the chloroform layer in the final stage is rapid and complete but it may at times be necessary to give a short centrifugation before reading to remove suspended chloroform droplets. The colour of the test solution is occasionally slightly different in shade from that of the standard. This is due to an orange compound with an absorption maximum at 480 mμ formed with some urines. Its formation is reduced to negligible proportions with the dilutions of urine extract and reagent used in the method. The vanillyl-mandelic acid complex may be freed from traces of this orange component by chromatography after acidifying the NaOH solution with HCl and extracting the resulting yellow colour into chloroform. The extracted material is adsorbed from the chloroform onto a column of magnesium oxide mixed with cellulose powder to aid solvent flow. The chromatogram is developed with methanolic KOH, and the orange component, if present, moves down the column ahead of the red vanillyl-mandelic acid band, which can then be eluted separately. Using this method the vanillyl-mandelic acid-diazo derivative from 250 ml. of urine has been separated and shown to have an optical absorption curve identical with that obtained with known vanillyl-mandelic acid carried through the same procedure and a mixture of standard and urine derivatives could not be separated on a magnesium oxide column. The infra-red spectra of the two coloured materials have also been compared and are essentially the same, confirming that the method is determining vanillyl-mandelic acid without interference from the many other urine constituents which couple with the diazo reagent.

Studnitz (1960) considered ether and ethyl acetate equally satisfactory for extracting vanillyl-mandelic acid from urine. We find that ether has a greater selectivity against unwanted colour-forming substances and is therefore preferable, although as vanillyl-mandelic acid has a considerably lower distribution coefficient in ether/water than in ethyl acetate/water fairly large volumes of solvent are needed for extraction. However, the bulk of the ether used is recovered for redistillation and further use. It is possible to remove the ether by distillation under reduced pressure but precautions must be taken to see that the ether used is completely free of peroxides as otherwise vanillyl-mandelic acid suffers considerable destruction. The extraction procedure avoids this difficulty besides taking less time.

RESULTS

The reproducibility of the coupling reaction and subsequent stages has been determined by analysis of 14 50μg. samples of standard vanillyl-mandelic acid solution. These were read at 510 mμ in a 1 cm. cell. The mean of the optical density readings was 0·348 with a standard deviation of ± 0·0063 (± 1·81%). Recoveries of 20 and 30 μg. amounts of vanillyl-mandelic acid added to normal urine were 95·0% and 95·9% respectively. Complete extraction could be obtained only by increasing considerably the number of ether extractions and it is more convenient to use a factor of 1·05 to correct for this loss. This correction has been applied to the figures quoted in Tables I and II. Table I gives the total vanillyl-mandelic acid excreted in 24 hr. by a number of normal male subjects. Subjects A and B were medical students on balance experiments and therefore on a complete but controlled diet and 24-hr.

TABLE I

EXCRETION OF VANILLYL-MANDELIC ACID BY NORMAL SUBJECTS

Subject	*Vanillyl-mandelic Acid Output (mg./24 hr.)*
A1	13·3
2	11·0
3	10·6
B1	10·0
2	10·6
3	9·0
C	15·0
D	17·9
E	9·0
F	17·0
G	12·9
H	11·1
I	17·6

TABLE II

EXCRETION OF VANILLYL-MANDELIC ACID BY SUBJECTS WITH PHAEOCHROMOCYTOMA

Subject	*Output (mg./24 hr.)*	
	New Method	*Sandler and Ruthven Method*
J 1	12·3	—
2	16·7	—
K1	14·8	12·8
2	23·7	20·0
L	25·6	27·5

urine samples on successive days were available and have been grouped in Table I. The other samples were from laboratory staff on unrestricted diets.

Table II shows pre-operative vanillyl-mandelic acid excretion figures for three proved cases of phaeochromocytoma, the urines being made available by the courtesy of Dr. M. Sandler. The two values given for subjects J and K were on urine samples obtained on different occasions. In the second column are the values supplied by Dr. Sandler for three of the urines using the method of Sandler and Ruthven (1959) as modified by Sandler and Ruthven (1960). Comparison of the two sets of figures shows reasonably good agreement between the two methods despite the very different techniques employed.

We thank Dr. M. Sandler for making available the phaeochromocytoma urine samples and for giving permission to quote his vanillyl-mandelic acid values for these urines. We also thank Dr. A. D. Munro-Faure for valuable discussion and for providing the urine samples from subjects A and B, and Dr. A. J. Everett for determining the infra-red spectra of the vanillyl-mandelic acid-diazo derivatives.

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Factors concerned in the efficient steam sterilization of surgical dressings

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SYNOPSIS Some of the factors affecting the efficient steam sterilization of dressings have been examined. A jacketed sterilizer will process a load more quickly than a sterilizer without a jacket. The level of fore-vacuum is critical and must reach an absolute pressure of 20 mm. Hg (29·2 in.Hg vacuum) or less. This will overcome all conditions of overpacking studied. The level of after-vacuum should be 100 mm. Hg absolute or less, preferably near 50 mm. Hg absolute.

Overpacking cannot be defined in terms of weight of a fabric per unit volume of container but occurs when a load is compressed in its container.

It is surprising that, despite the recent important changes in design of steam sterilizers, little critical published work has appeared relating to the principles underlying these changes. The high pre-vacuum, high-pressure sterilizer is now regarded as the best steam sterilizer available and yet there is no substantial experimental work to encourage the use of steam at a pressure of 30 to 32 pounds per square inch (p.s.i.); indeed doubts have been expressed as to the wisdom of using this pressure as opposed to lower levels (Knox, 1961). Since the original work of Knox and Penikett (1958) on the level of fore-vacuum to be employed in high pre-vacuum sterilization no detailed confirmation of their results has been presented, although suggestive supportive evidence is available (Magee and Oakes, 1958; Alder and Gillespie, 1959; Wells and Whitwell, 1960).

A high pre-vacuum contributes to the efficient steam sterilization of dressings, *i.e.*, a process which produces a dry, sterile load in the shortest possible time without damage to the load. Other factors which contribute to efficiency are effective drying of the load with which must be bracketed a good supply of dry, saturated steam, prevention of the load being recontaminated by unsterile air at the end of the process, and finally any modification to a sterilizer which increases its rate of turnover.

In the present study the factors concerned with the efficiency of such sterilizers which have been considered are: Speed of sterilization in relation to whether a sterilizer should be jacketed or unjacketed, effective removal of air by high pre-vacuum, and drying of a load. Finally, as downward-displacement sterilizers are still widely used some studies have been made on the efficiency of the downward-displacement method of removing air.

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THE EXPERIMENTAL STERILIZER

The sterilizer used was a cylindrical, horizontal dressings sterilizer of 4·2 cubic ft. capacity. Steam was supplied from the hospital main supply at a pressure of 60 to 65 pounds per square inch gauge (p.s.i.) and passed through a separator to a reducing valve which reduced the pressure to 20 p.s.i. The steam was supplied at this pressure to the jacket and chamber independently. Steam entered the chamber at the back and was deflected by a baffle plate. The chamber drain was situated at the front of the chamber and served also as the point from which the vacuum was drawn by a steam jacketed, oil-sealed, air-ballasted pump. A steam ejector was also fitted. This drew its vacuum through the steam-to-chamber steam pipe.

The oil-sealed vacuum pump, which was designed to pump water vapour and air, was connected to the chamber drain by a pipe which was heated to prevent steam from condensing in it before reaching the pump. The pump usually took about five minutes to reduce the chamber pressure from atmospheric to 20 mm. Hg absolute pressure. The chamber drain led through a hand-operated valve, a non-return valve, and a balanced-pressure thermostatic steam trap to a tundish.

INSTRUMENTATION

TEMPERATURE Fourteen metal-sheathed, copper constantan thermocouples 1/16 inch (0·159 cm.) in diameter were led through the sterilizer door. It was found that they could be threaded through a silicone rubber bung held in

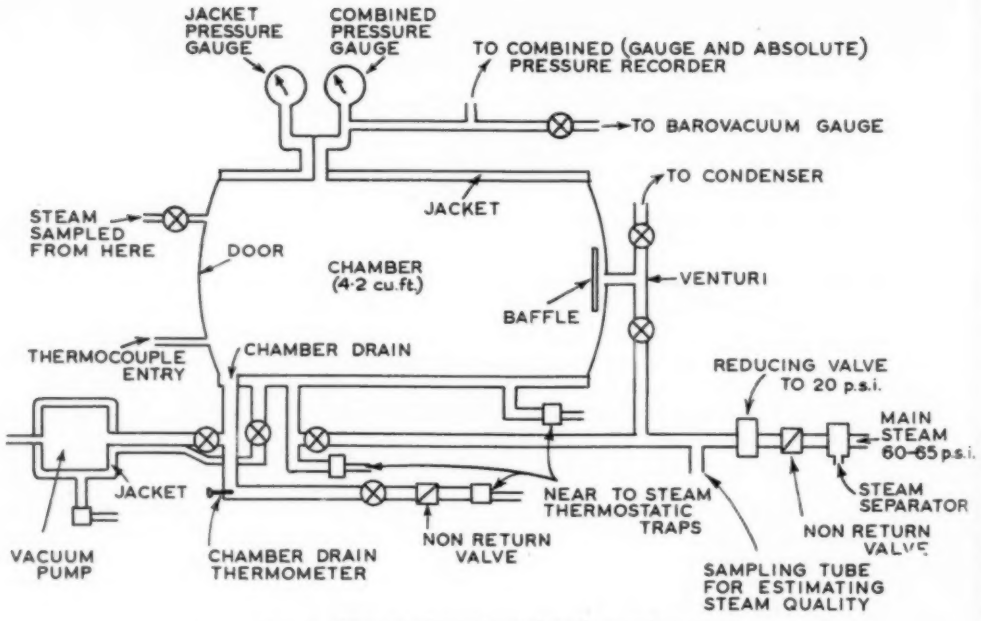

FIG. 1. *Diagram of experimental sterilizer and pump.*

a valve gland screwed into the door without causing any vacuum leakage. Another thermocouple was inserted into the chamber drain line 12 in. from the chamber. These thermocouples were connected to a 16-point high-speed electronic recorder. The temperature registered by each thermocouple was printed on a chart, there being a five-second interval between each successive recording. Hence the temperature at any single point was recorded once every 80 seconds. The recorder was calibrated from 30° to 140° C. in 2°C. divisions, the overall accuracy being 0·5%. In practice the temperature was read to the nearest degree Centigrade except during calibration runs.

The thermocouples were calibrated from time to time against a mercury-in-glass thermometer using an oil bath. Their speed of response was such that a full-scale deflection could be obtained inside the five-second interval between the printing of the recorder. The effect of heat conduction along the thermocouples leads on the accuracy of recording was found to be negligible.

PRESSURE AND VACUUM RECORDING Jacket pressure was indicated on a Bourdon gauge. The chamber pressure was indicated on a combined pressure and vacuum gauge and was recorded on a combined gauge and absolute-pressure recorder. The degree of vacuum was also ascertained by a mercury baro-vacuum gauge. This latter gave a direct reading in millimetres of mercury absolute pressure and was therefore independent of variations in barometric pressure.

LOADS Most experiments were carried out using 20 in. × 30 in. cotton huck towels, each folded into 12 thicknesses. For most experiments these towels were placed, in varying numbers, inside cylindrical dressing drums (10 in. diameter × 9 in. deep), which were lined with a further two such towels in order to simulate drum liners. The drums had 14 groups of 16 holes 5/32 in. in diameter spaced round the circumference.

Thermocouples were introduced into the loads in such a way that when the drums were placed in the sterilizer the thermocouples entered from the bottom of the drum, *i.e.*, in the opposite direction to that from which steam would enter the load by downward displacement of air. The delay in temperature rise seen in many experiments suggested that steam did not track alongside the thermocouples.

CONFIRMATION OF DEGREE OF VACUUM NECESSARY FOR RAPID STEAM PENETRATION INTO A LOAD

For the purposes of comparing the effect of different degrees of fore-vacuum on steam penetration, a load which was only slowly penetrated by steam by the downward displacement method was used. This, it was thought, should show well any delay in steam penetration due to an inadequate fore-vacuum. The load chosen consisted of 16 towels (plus a further two lining the drum), each folded into 12 thicknesses and so placed that the layers of towel lay in a horizontal plane when the drum was pos-

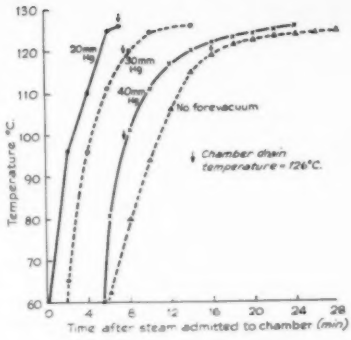

FIG. 2. *Effect of different degrees of fore-vacuum on temperature rise in coolest part of load after admission of steam to chamber.*

tioned correctly, *i.e.*, on its side, in the sterilizer. Thermocouples were introduced from the bottom of the load as positioned in the sterilizer and were placed in the same position in each experiment.

In these experiments the chamber was evacuated to the desired extent, steam was admitted, and the temperature rise in different parts of the load was noted. The slowest rises in temperature in experiments using different degrees of fore-vacuum are shown in Fig. 2.

It can be seen that, with a fore-vacuum giving a pressure of 20 mm. Hg absolute, the temperature inside the load rose rapidly once steam was admitted to the chamber, and reached 126°C. (the temperature of saturated steam corresponding to the gauge pressure of 20 p.s.i.) at the same time as the chamber drain thermometer. With a fore-vacuum of only 30 mm. Hg absolute, however, there was a delay in steam penetration and with a fore-vacuum down to 40 mm. Hg absolute (28·4 in. Hg vacuum at a barometric pressure of 30 in. Hg—a level of vacuum offered by some sterilizer manufacturers) the time taken for steam to penetrate the load differed but little from that taken by downward displacement.

The critical nature of the level of fore-vacuum necessary to obtain almost instantaneous steam penetration into a load was thus demonstrated. Further experiments showed that even with a load of 29 folded towels where the drum lid had to be forced shut, a fore-vacuum down to 20 mm. Hg absolute enabled steam to penetrate the load instantaneously.

These findings confirm the conclusions of Knox and Penikett (1958). The method used in the present observations differed from that used by Knox and Penikett in that the time required to reach saturated steam temperature in the load was examined on each occasion instead of that required to reach an arbitrary temperature of 115°C. Both sets of observations agree, however, and make it clear that any high-vacuum sterilizer which fails to achieve a fore-vacuum down to at least 20 mm. Hg absolute pressure is potentially dangerous, because if the sterilization process is timed from the chamber drain temperature the cycle may be completed before the load has been penetrated by steam.

Apart from inability to draw an adequate fore-vacuum the high vacuum sterilization process may be defeated by a rather more subtle fault.

If air can leak into the chamber between the time when the vacuum pump is disconnected from the chamber and the steam pressure reaches zero gauge, steam penetration into the load may be slow (Fallon, 1961a). This fault will tend to be obscured by a powerful vacuum pump which will overcome small leaks and will be revealed only by monitoring of a load in the sterilizer either by Browne's tubes or thermocouples. It might be revealed by a careful inspection of the chart showing the time taken to reach the final level of fore-vacuum. Hence a daily inspection of charts from such a sterilizer and the immediate investigation of any deviation from normal is highly important, as is emphasized in the M.R.C. report (1959).

THE DEGREE OF AFTER-VACUUM

Dressings after sterilization can be rapidly and effectively dried merely by drawing an after-vacuum down to 50 mm. Hg absolute (Penikett, Rowe, and Robson, 1958). However, these authors state that in their experiments the load achieved a moisture content of less than 5% and was by their definition 'dry' when the pressure in the chamber was reduced to about 300 mm. Hg (18 in. of vacuum) with a single drum or 200 mm. Hg (21 in.) with a full load of dressings without the vacuum being held. As they note, this level of after-vacuum may readily be obtained in many sterilizers at present installed in hospitals. It is evident that if merely drawing an after-vacuum of the order of 18 in. Hg without any airing of the load will give dry dressings, the dangers of recontamination of a load associated with prolonged airing through inadequate filters and of damage to the load due to prolonged contact with hot air would be avoided. In view of the importance of this subject the findings of Penikett *et al.* were re-examined.

In any experiments on the moisture uptake of dressings it is important that the steam entering the chamber should be of good quality. The quality of steam supplied to the experimental sterilizer was measured at two sites by different methods.

THROTTLING CALORIMETRY The quality of steam in the 20 p.s.i. supply line was measured by this method. Steam was sampled by means of a horizontal pipe, of the same diameter as the steam line, leading to a diaphragm in the centre of which was a hole 1/64 in. diameter (0·0397 cm.). The dryness fraction of the steam could be calculated from the temperature of the steam issuing from this hole to atmospheric pressure. The steam in the supply line had a dryness fraction of 0·98.

BUCKET CALORIMETRY The method used was similar to that described by Penikett *et al.* (1958) except that the steam in the chamber was sampled through a hole bored in the sterilizer door, the steam being led through a well-lagged tube to a Dewar flask. The steam sampled from

this site may well have given a lower dryness fraction than that which actually obtained in the chamber, due to the continual condensation of steam when it met the relatively cool door with the possible formation of some water fog at that point. The quality of the steam was measured with loads actually in the chamber. Eleven estimations gave a mean value of 0·90 (standard deviation 0·04).

The discrepancy between the two results suggests that despite lagging of the steam line some condensation had taken place in the steam before it entered the chamber but the fact that steam was sampled from the door and in the presence of a load would also play a part.

EXPERIMENTAL

Loads to be sterilized consisted of cotton huck towels placed in the dressing drum so that the folds lay in a vertical plane if downward displacement sterilization was being studied. In high vacuum studies the drums were placed on their base to reproduce the conditions used by Penikett *et al.* (1958).

Towels were sterilized in drums or packs and were weighed, immediately before and after sterilization, on a balance which could be read to the nearest gram. Between experiments the towels were placed on a rack and their moisture content was allowed to equilibrate with that of the air in the hospital. Before any run the relative humidity of the atmosphere was determined using a wet- and dry-bulb thermometer. The relative humidity ranged from 36% to 77%, an average level being about 53%. There was no constant relationship between the relative humidity and the weight of towels on any one day.

It was found that the moisture gain of a load was always greater on the first sterilizing run of the day so that all experiments reported here were carried out after the sterilizer had been used for one or more runs so that the door and back of the sterilizer as well as the steam lines were thoroughly hot.

Sterilizing runs were carried out with and without a preliminary fore-vacuum. In most cases the steaming period was for 10 minutes after the chamber pressure reached 20 p.s.i. but in some experiments longer periods of steaming were carried out to see if this had any effect on the moisture content of a load. Some experiments were carried out to see whether the residual moisture content was related to the type of container used. At the end of the steaming period different degrees of after-vacuum, produced rapidly by the oil-sealed pump, were applied. The vacuum was broken rapidly (in less than 30 sec.) as soon as the appropriate level was reached and the load was then removed from the sterilizer and container and was weighed immediately. Errors due to the temperature of the load would in fact be negligible and it was also observed that the loss of moisture through evaporation was not so rapid as to render accurate weighing difficult because the balance used was well damped and read almost instantaneously.

RESULTS

The results using eight cotton huck towels (plus two

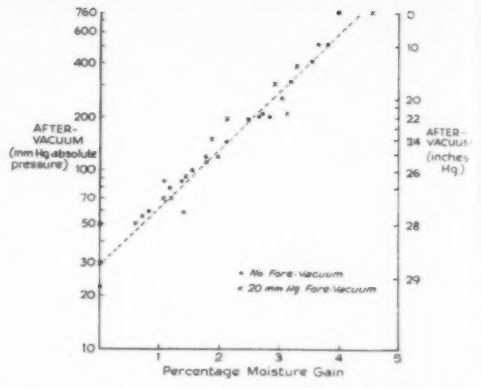

FIG. 3. *The relationship between after-vacuum (log scale) and percentage moisture gain is approximately linear.*

lining the drum) are shown in Fig. 3 in which the percentage moisture gain is plotted against the after-vacuum applied. It can be seen that although there was some scatter in the results obtained by weighing, the relationship between the degrees of after-vacuum (plotted logarithmically) and the percentage moisture gain was roughly linear and that if the final absolute pressure reached during the vacuum phase was above 100 mm. Hg (26 in. Hg vacuum) the moisture gain would generally be greater than 1·5%. There was little difference whether a high pre-vacuum or downward-displacement method of sterilization was used.

With the towels used in these experiments it was found that their moisture content averaged about 4·5% before sterilization so that a gain in moisture of over 1·5% would mean that the towels would be subjectively damp (Penikett *et al.*, 1958). Experiments with tightly packed drums containing 20 to 25 towels showed that the moisture gain was only slightly higher than that in loosely packed drums but that in the few experiments conducted with packs of eight towels wrapped in two similar towels the percentage moisture gain was much less than with drums. Loads steamed for longer than 10 minutes at 20 p.s.i. did not show a higher moisture gain than those steamed for 10 minutes. The weighing of a complete load is much more realistic than that of weighing towelling samples placed in the load as it gives some indication of the condition in which the whole load will be on delivery to the user. It is little comfort to either the surgeon or the bacteriologist if the centre of a load is well dried but the outer layers are wet and therefore more vulnerable to bacterial penetration. The results obtained therefore confirm that once a pressure of 50 mm. Hg absolute is reached the drying phase can be ended but they suggest that vacua of less than 26 in. Hg, *i.e.*, 100 mm. Hg absolute, will produce a damp load if the dressings are contained in a drum.

THE EFFECT OF A HEATED JACKET ON EFFICIENCY OF STEAM STERILIZATION

From time to time doubts have been cast on the advantages of having a steam-heated jacket round the chamber of a sterilizer. In the past such a jacket has probably acted as a steam separator where the chamber steam has been supplied from the jacket, but with a modern installation the steam supply should be dry before it reaches the sterilizer (M.R.C. report, 1959). A jacket increases the cost of a sterilizer and will be a source of steam wastage where there are long intervals between sterilizing cycles. Also where sterilization cycles follow one another rapidly it might be argued that a lagged chamber would be satisfactory as the walls would hold a considerable amount of heat. In order to see what would be the effect of the absence of a heated jacket on the performance of a sterilizer the experimental model was run with an unheated jacket. This would probably not be as efficient as a well-lagged chamber but would give some indication of what might be expected in a sterilizer without a jacket compared with the same sterilizer with a heated jacket.

Runs with the jacket cold were compared with similar runs where the jacket was heated. A load of 16 towels was used, the towels being packed so that they were in a vertical plane, parallel to the direction of steam entry. The results are shown in Fig. 4.

It can be seen that starting from cold even a high-vacuum run was very slow, the load heating up less rapidly than that in a downward-displacement run with a heated jacket.

Even after the chamber had been heated by previous use there was a substantial delay in the heating up of a load compared with that when the jacket was heated. Jones and Finney (1960) noted that with a large non-jacketed sterilizer difficulty was experienced in drying the load until cast iron plates were placed in the sterilizer to provide more stored heat for the drying of dressings. Certainly during the drying part of the cycle and when the sterilizer is being unloaded and reloaded the chamber walls will cool so that only part of the heat taken up by the wall in one run will be available for the next.

The results suggest that a jacket is a good feature even in a high-vacuum sterilizer, except perhaps in those which are used infrequently and then with no degree of urgency.

EFFICIENCY OF DOWNWARD-DISPLACEMENT STERILIZATION

Downward-displacement sterilization has been the only method of steam sterilization (often used in conjunction with a partial fore-vacuum) in use in Britain until the recent introduction of high-vacuum sterilizers. However, many so-called sterilizers were faulty when examined (Bowie, 1955; Howie and Timbury, 1956) and it is only in recent times that many of these have been upgraded as suggested by Scott (1957). In correctly designed sterilizers, as noted by Walter (1948) and Perkins (1956), downward displacement of air works satisfactorily provided care is taken to pack the load so as to facilitate steam penetration. This is important as the difference in the specific gravity of air and steam is slight and the resulting force of

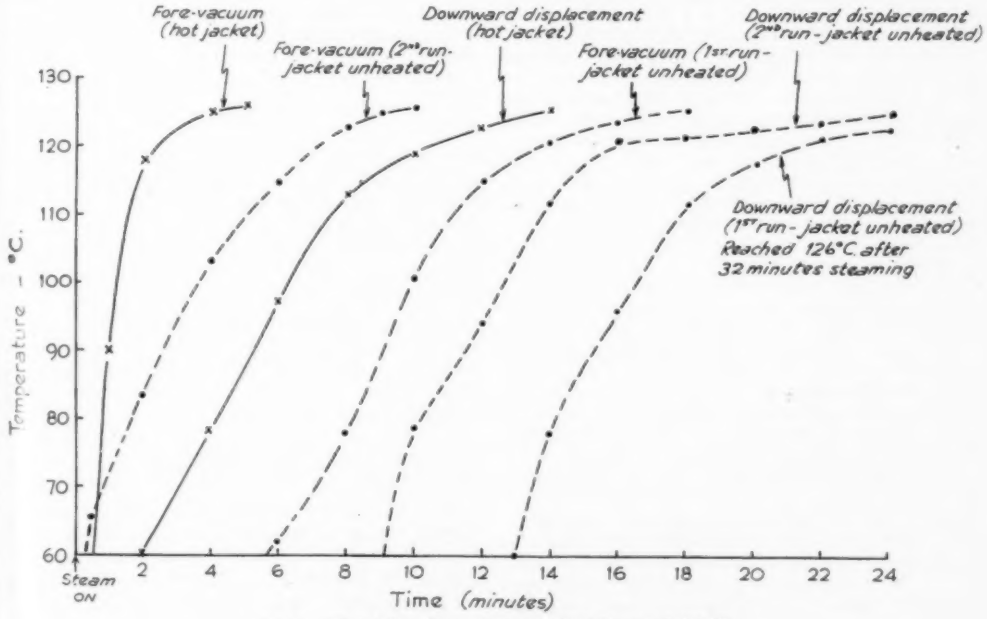

FIG. 4. *Time taken for coolest part of load to reach 126°C.*

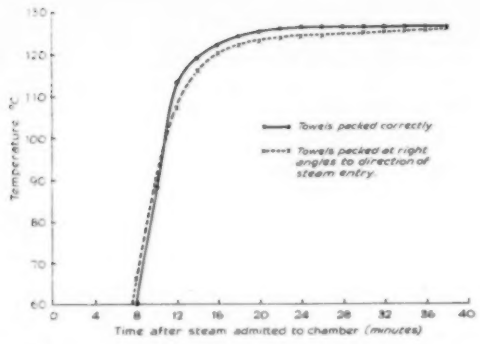
Towels packed correctly
Towels packed at right angles to direction of steam entry
Temperature °C
Time after steam admitted to chamber (minutes)

FIG. 5. *Delay in steam penetration due to air pockets caused by incorrect position of load.*

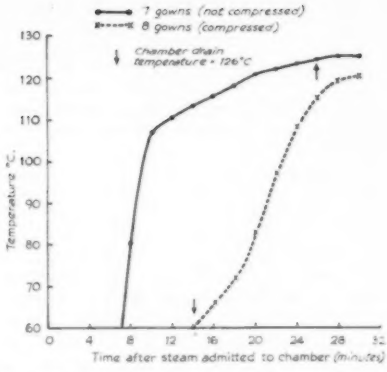
7 gowns (not compressed)
8 gowns (compressed)
Chamber drain temperature - 126°C
Temperature °C
Time after steam admitted to chamber (minutes)

FIG. 6. *Effect of compression of load on steam penetration. The temperature shown is that of the coolest part of the load.*

downward displacement is weak (M.R.C., 1959). It is easy to defeat downward displacement by, for instance, placing a drum on its base so that the holes for steam entry and air removal are at the side. Even the mere act of placing towels so that they lie in a horizontal rather than a vertical plane when placed in the sterilizer can delay steam penetration (Fig. 5). It is, however, not certain what will defeat downward displacement if the load is orientated correctly. An attempt was made, therefore, to define what load in a dressing drum, *i.e.*, what degree of over-packing, would defeat downward-displacement sterilization. For this purpose different loads of cotton goods (towels or surgical gowns) were placed in a 10 in. × 9 in. dressing drum and the time taken for the temperature to rise to 126°C. was noted. When a weight of cotton was found which would prevent steam penetration so that the load would be at a temperature of 126°C. for less than 10 of the 30 minutes allowed from the time the chamber drain temperature reached 126°C. (the time recommended for the sterilization of dressings in drums in the M.R.C. report, 1959), an attempt was made to see how a similar weight of cotton wool would behave. It was found, however, that only about a third to one half of the weight of cotton wool could be packed into the drum and that this grossly delayed steam penetration. Hence it does not seem possible to define, in grams per c.c., what is an over-packed drum for cotton fibre. However, it was observed that quite full drums could be penetrated by steam but that as soon as a load was compressed on closing the drum lid steam penetration was seriously delayed (Fig. 6). Not only does such a delay mean that the right temperature is either reached slowly or not at all but it indicates the possibility of air pocketing, so that even if a temperature of 126°C. was reached ultimately, there is a possibility that this may be inside a pocket of air which has reached the temperature of its surroundings. Therefore, all that can be said is that as long as a load of dressings is correctly orientated and not compressed, steam will penetrate it. These results were checked in a larger (9 c. ft.) sterilizer with a larger (10 in. × 14 in.) dressing drum and there it was found that even when the towels were stacked one on top of another leaving a free space between them and the drum walls, if the pile was compressed by the drum lid, penetration was delayed. The delay in penetration was as much as 15 minutes compared with a loosely packed drum. Conditions in a loosely packed drum were reflected well by the chamber drain thermometer, but, as found in experiments with different degrees of fore-vacuum, a chamber drain thermometer will not always give an indication of slow penetration of steam into a load.

DISCUSSION

The most important finding in this study has been the confirmation of the work of Knox and Penikett (1958) on the critical nature of the level of fore-vacuum which must be drawn in a high-vacuum sterilizer in order to accomplish almost instantaneous steam penetration into a load. Where a temperature of 126°C. is employed for sterilization, slight delay in steam penetration might be serious (using a 10 minutes' holding period) but where a temperature of 134°C. is used (three minutes' holding period) the slightest delay will almost certainly seriously impair the sterilization process. It is important therefore that not only should a high-vacuum sterilizer be specified to produce a fore-vacuum giving an absolute pressure of 20 mm. Hg or less but that before it is accepted for use it must be tested with an accurate vacuum gauge to ensure that it is producing this vacuum. The incorporation of a pressure-stat to prevent the sterilization cycle from proceeding until the correct level of vacuum has been reached is a commendable feature of commercial models available,

but periodic tests of a sterilizer should be designed to check on the accuracy of this component. The danger of an air leak which would allow air to enter the chamber after the fore-vacuum had been drawn is a real one and there is no doubt that the best way of countering such a possibility is to control the sterilization process by means of a monitored test drum. Such a test drum is described elsewhere (Fallon, 1961a) but the essential feature of it is that it cannot easily be penetrated by steam using downwards displacement and will therefore show at once any failure in the high-vacuum process. The importance of an adequate fore-vacuum cannot be overemphasized.

As stated in the second communication of the M.R.C. working party on pressure steam sterilizers (1960), high-vacuum sterilizers are, in general, proving satisfactory in practice. From the point of view of increased efficiency in speed of sterilization this is well described by Magee and Oakes (1958) and Alder and Gillespie (1959) where downward-displacement machines have been converted to high pre-vacuum working and by Wells and Whitwell (1960) with a small prototype high-speed, high-vacuum sterilizer.

The degree of after-vacuum is of some importance as the method of producing the vacuum depends upon the level required to dry dressings without any airing period. The findings here suggest that the system employed should be able to produce an after-vacuum to give a pressure of the order of 100 mm. Hg absolute or better.

The difference in the results obtained by Penikett *et al.* and those in this study are almost certainly due to the way in which the moisture content of the load was measured. In their work Penikett *et al.* measured the moisture content of towel samples which were always placed in exactly the same position (Penikett, personal communication), whereas I was concerned to measure the total moisture gain of a load in the belief that the total moisture content (which will be irregularly distributed throughout a load) is what matters to the user and also in the prevention of bacterial penetration of a load.

It is interesting to note that packs of dressings dry out more easily than dressings in drums. This is yet another good reason for using wrapped packs rather than dressing drums.

From the point of view of efficiency of sterilization there seems to be little doubt that a jacketed sterilizer has advantages over an unjacketed model and the small initial extra outlay will be well justified in terms of increased output of such a machine. Also condensation difficulties of the nature of those reported by Jones and Finney (1960) will be less in a jacketed sterilizer. (A number of commercial jacketed models do have a small pool of condensate lying on the bottom of the chamber after a cycle. This may be due to wet steam, an inner liner to the chamber which prevents all the condensate from reaching the hot chamber wall, or perhaps due to a chamber shape which does not aid the condensate to flow to the chamber drain.)

In downward-displacement sterilizers, steam penetration through a badly packed load will be delayed. Although bad packing cannot be defined precisely in measureable terms, users of such sterilizers must not only orientate the load correctly in relation to steam flow but must not in any way compress the contents of a dressing container. Here again wrapped packs have a great advantage over other containers in that they cannot easily be tightly compressed. They may, however, be of too great a size or have articles incorporated in them which would interfere with steam penetration (Perkins, 1956) and care must be taken to avoid these dangers. Some types of container used for surgical dressings may delay steam penetration when used in a downward-displacement sterilizer (Fallon, 1961b).

In any discussion of the efficiency of steam sterilization mention must be made of how to prevent recontamination of dressings. It is therefore relevant to re-emphasize the importance of the findings of the M.R.C. working party (1960) that spun glass filters are superior to those in current use on most sterilizers.

I am grateful to the Department of Health for Scotland for the sterilizer shell; the Board of Management for the Western Infirmary, Glasgow, for a grant from their endowment funds towards the cost of equipment; to Mr. J. Wood, hospital engineer, and his staff, for their careful installation and maintenance of the sterilizer; to Dr. E. A. Bruges, Senior Lecturer in Mechanical Engineering, University of Glasgow, for advice on steam quality measurement, and to Mr. R. Callander for the diagrams.

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A case of meningitis due to *Listeria monocytogenes*

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SYNOPSIS A case of meningitis due to *Listeria monocytogenes* is reported. The source and mode of infection could not be ascertained. The patient recovered after treatment with chlortetracycline.

Listeria monocytogenes was first described in 1926 as the cause of an epizootic in laboratory animals in Cambridge (Murray, Webb, and Swann, 1926). Although by 1958 approximately 500 human infections with this organism had been recorded (Seeliger, 1958) there are relatively few such reports from Great Britain. The following case of listerial meningitis is therefore presented.

CASE REPORT

H.T., an estate agent aged 39, was admitted on 22 June 1960 with a history of headache and vomiting for 12 hours. His temperature was 102°F., he was drowsy, had neck rigidity and a positive Kernig's sign. Cerebrospinal fluid (C.S.F.) taken on admission was under pressure of 220 mm. H_2O and contained 66 cells per c.mm. (predominantly lymphocytes), 50 mg.% protein, and 62 mg.% sugar. No organisms were seen in direct Gram and Ziehl-Neelsen films and cultures remained sterile. A blood count showed 21,200 white cells (88% polymorphs, 11% lymphocytes, and 1% monocytes). The E.S.R. was 8 mm. in one hour.

In view of the C.S.F. findings a presumptive diagnosis of virus meningitis was made and the initial treatment with six-hourly injections of 2 mega units of penicillin and six-hourly doses of 1·5 g. sulphadiazine by mouth was discontinued after 24 hours. As the patient's drowsiness and pyrexia persisted a second C.S.F. specimen was taken on 28 June 1960. It was under a pressure of 300 mm. H_2O and contained 217 cells per c.mm. (75% lymphocytes and 25% polymorphs). Protein was 220 mg. %, sugar 91 mg. %, chlorides 562 mg. %, and the Lange test was normal. Gram and Ziehl-Neelsen films were again negative, but a subculture of the C.S.F. grew a Gram-positive organism which was later identified as *Listeria monocytogenes*. A course of six-hourly chlortetracycline, 250 mg., was now started and under this treatment, which continued for two weeks, the patient's condition slowly improved. Pyrexia subsided within three days and neck stiffness and drowsiness gradually diminished. The white cell blood count which on 29 June had still been 16,000 (82% polymorphs, 14% lymphocytes, and 4% monocytes) decreased by 21 July to 11,500 (74% polymorphs, 1% eosinophils, 22% lymphocytes, and 3% monocytes). A third specimen of C.S.F. on 17 July 1960 still contained 153 cells per c.mm., 89% of which were lymphocytes; protein was 140 mg. %, sugar 67 mg. % and chlorides 644 mg. %. The Lange test gave a paretic type curve: 5554100000. Cerebrospinal fluid and blood Wassermann reactions were negative and culture of C.S.F. remained sterile.

Specimens of C.S.F., faeces, and a throat swab sent to the Routine Diagnostic Laboratory, Colindale, for virus isolation, and paired sera for routine virus complement-fixation tests all gave a negative result.

The patient made a complete recovery and was discharged on 26 July 1960.

DESCRIPTION OF PATIENT'S (HT) ORGANISM

MORPHOLOGY AND CULTURAL CHARACTERISTICS The organism was a short, Gram-positive bacillus, 0·5 × 1 to 2 μ, occurring singly or in V-shaped or parallel pairs. It was motile when grown at room temperature. On horse blood agar it grew in small, smooth, transparent colonies, with an entire edge which after 48 hours attained a diameter of 1 to 2 mm.; the colonies were surrounded by a narrow zone of faint haemolysis. It was sensitive to discs containing penicillin (0·5 units), streptomycin (20 μg.), chloramphenicol (40 μg.), tetracycline (10 μg.), erythromycin (1 μg.), oleandomycin (2 μg.), ristocetin (25 μg.), neomycin (60 μg.), sulphafurazole (0·1 mg.), and furadantin (10 mg.). It was resistant to novobiocin (2 μg.) and to neomycin (30 μg.).

BIOCHEMICAL CHARACTERISTICS The biochemical reactions were compared with those of a stock culture of *Listeria monocytogenes* (N.C.T.C. 7973) obtained from the National Collection of Type Cultures (Table I).

VIRULENCE Four mice were injected intraperitoneally with 0·3 ml. of an overnight glucose broth culture diluted 1 : 10 of the patient's strain. A culture of tail blood of one of these mice taken 24 hours later yielded a profuse growth of the infecting organism. Three mice died, three days, and the fourth one, six days after, the injection, with evidence of septicaemia and slight peritonitis. In each of these, the organism was recovered from heart blood,

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TABLE I

BIOCHEMICAL REACTIONS OF CULTURES HT AND NCTC 7973

*Fermentation Reactions**	*HT*	*NCTC 7973*	*Other Biochemical Tests*	*HT*	*NCTC 7973*
Arabinose	–	–	Methyl red	–	–
Xylose	–	–	Voges-Proskauer	–	±
Rhamnose	+[3]†	+[3]	Citrate utilization	–	–
Glucose	+[1]	+[1]	Nitrate reduction	–	–
Laevulose	+[1]	+[1]	Gelatine liquefaction	–	–
Sucrose	+[6]	+[9]	H_2S production	–	–
Maltose	+[1]	+[1]	Indole production	–	–
Lactose	+[4]	+[3]	Urease production	–	–
Trehalose	+[1]	+[1]	Growth on McConkey agar	+	+
Raffinose	–	–	Growth on Hoyle's tellurite agar	+	+
Dextrin	–	+[9]	Growth on 7% salt agar	+	+
Inulin	–	–			
Glycerol	+[8]	±[8]			
Adonitol	+[10]	–			
Mannitol	–	–			
Dulcitol	–	–			
Sorbitol	–	+[10]			
Salicin	+[8]	+[8]			
Aesculin	+[1]	+[1]			
Inositol	–	–			

*Acid without gas.
†Day on which fermentation was first observed.

liver, and spleen. Sections of liver revealed numerous scattered micro-abscesses with occasional central necrosis containing many small Gram-positive bacilli. Similar confluent abscesses containing masses of bacilli were seen in the spleen. The kidneys showed only a few minute foci of infiltration with polymorphs and macrophages in the cortex in which Gram-positive bacilli could be found. No changes were seen in the lungs except for a massive infiltration of the visceral pleura with Gram-positive bacilli.

The instillation of three drops of an overnight glucose broth culture into the conjunctival sac of a guinea-pig and a rabbit produced within 24 hours an intensive conjunctivitis which subsequently became purulent and was associated with dense corneal opacities.

A rabbit injected intravenously with 1 ml. of an overnight glucose broth culture diluted 1 : 10 died after seven days with evidence of septicaemia. Necropsy revealed a sero-fibrinous pericarditis and multiple small abscesses in the spleen. The infecting organism was recovered from lung, spleen, and pericardial fluid.

Two other rabbits were injected intravenously, each with 0·25 ml. of overnight glucose broth cultures diluted 1 : 10 of the patient's organism (HT) and the type strain NCTC 7973, respectively. They survived and gave the blood counts shown in Table II.

The cells counted under the heading 'monocytes' showed a morphology which was intermediate between that of typical monocytes and large lymphocytes. They had a round, oval, or horseshoe-shaped nucleus of loose texture and abundant grayish blue cytoplasm. Both cultures produced the maximum monocyte response—25·5% with culture HT and 44·5% with culture NCTC 7973—on the third day after injection.

SEROLOGY Antisera were prepared by repeated intravenous injection of rabbits with formolized suspensions of cultures HT and NCTC 7973. These suspensions were also used for agglutination tests which were read after two hours' incubation at 37°C. and overnight stay at 4°C. For absorption, sera were diluted 1 : 25; each serum was absorbed three times with the packed deposit of a heavy formolized suspension of the absorbing strain. Table III shows that each strain absorbed completely its autologous antibody and antibody reacting with the other

TABLE II

BLOOD COUNTS OF RABBITS INJECTED WITH CULTURES HT AND NCTC 7973

Day after Injection	*Rabbit Inoculated with*							
	Culture HT				*Culture NCTC 7973*			
	Total White Count	*Polymorphs (%)*	*Lymphocytes (%)*	*Monocytes (%)*	*Total White Count*	*Polymorphs (%)*	*Lymphocytes (%)*	*Monocytes (%)*
Before	9,000	33	63	4	5,700	68·5	26·5	5
1	7,500	65	32	3	4,600	58	40	2
2					4,800	58·5	30	11·5
3	8,300	57	17·5	25·5	9,000	30·5	25	44·5
4	9,400	65·5	17·5	17	5,000	38	34·5	27·5
5	9,500	40·5	43·5	16				
6	9,400	53	27·5	19·5	7,400	40·5	27·5	32
7	11.200	56	31	13	6,500	39·5	32·5	28

strain, both from its own antiserum and from antiserum prepared with the other strain.

TABLE III

ABSORPTION OF ANTISERA WITH CULTURES HT AND NCTC 7973

Antiserum against Culture	*Absorbing Antigen*	*Agglutinable Antigen*	
		Culture HT	*Culture NCTC 7973*
HT	—	12,800	6,400
	Culture HT	0	0
	Culture NCTC 7973	0	0
NCTC 7973	—	1,600	6,400
	Culture HT	0	0
	Culture NCTC 7973	0	0

DISCUSSION

Except for the negative Voges-Proskauer reaction, the absence of late fermentation of dextrin and sorbitol, and the presence of late fermentation of adonitol, the biochemical reactions of the patient's organism were identical with those of the type strain of *Listeria monocytogenes*. Both cultures produced an increase of abnormal mononuclear cells in the blood of rabbits, and absorption tests on antisera prepared with them showed their close antigenic similarity. The virulence of the patient's organism for mice on intraperitoneal inoculation, and for rabbits and guinea-pigs on intra-ocular instillation, further confirmed the organism as *Listeria monocytogenes*.

Repeated examinations of the patient's serum during and after his illness failed to reveal any agglutinating antibodies against a formolized suspension of his own organism. This may have been due, as was pointed out by Seeliger (1958), to lack of antigenic stimulus as the result of antibiotic treatment.

The source and mode of infection of this patient are unknown. Listeriae have been found in more than 30 species of animals, including cattle, sheep, pigs, cats, dogs, and a variety of rodents and birds (Gray, 1958). There was no history of close contact with any of these animals. Nor did the patient remember having drunk any unpasteurized milk, which according to Dediė (1958) and de Vries and Strikwerda (1956), may contain this organism. The mode of infection with Listeriae remains in most cases obscure. There are only a few observations in which there is strong circumstantial evidence of transmission from animals to man. Ödegaard, Grelland, and Henriksen (1952) described a fatal case of listerial meningitis in a farmer who fell ill after cleaning a stable containing sheep infected with this organism. And van der Schaaf (1955) observed another farmer who developed fatal listerial meningitis after assisting an aborting cow from a herd in which cases of listerial encephalitis were subsequently found.

Human listerial infection manifests itself in various forms. The most frequent one is a septicaemic condition of the foetus or newborn known as granulomatosis infantiseptica which is responsible for a proportion of spontaneous abortions and neonatal deaths. According to Potel (1958), who investigated 247 bacteriologically proved cases of listeriosis, 75% belonged to this group. The second most frequent manifestation is listerial meningitis or meningo-encephalitis which occurs in both infants and adults; and the third one is the adenoid form which presents as glandular fever. Except for one case of this last type reported by Webb (1943), all listerial infections reported in Great Britain have been meningeal (Gibson, 1935; Wright and McGregor, 1939; Mair, Mair, Stirk, and Reid, 1957; Edmunds, Nicholson, and Douglas, 1957; Barrow and Pugh, 1958; Turner, Dawson, and Pryce-Jones, 1958; Moore and Whitmore, 1960). It is possible that infections with this organism are more frequent than is generally supposed. Since the completion of this investigation five more cases of meningeal infection have been recorded in the weekly reports of the Public Health Laboratory Service (McDonald, 1961). In view of the above-mentioned frequency of neonatal listeriosis it is likely that systematic bacteriological investigation of neonatal deaths would reveal further instances of this infection in Great Britain.

I wish to thank Dr. R. Green for permission to quote from the case history, Professor R. A. Webb for confirmation of the bacteriological diagnosis, Dr. J. C. McDonald for data from the weekly reports of the Public Health Laboratory Service, and Mr. O. Stout for technical assistance.

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Disinfection of woollen blankets in steam at subatmospheric pressure

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SYNOPSIS Blankets may be disinfected in steam at subatmospheric pressures by temperatures below boiling point inside a suitably adapted autoclave chamber.

The chamber and its contents are thoroughly evacuated of air so as to allow rapid heat penetration, and steam is admitted to a pressure of 10 in. Hg below atmospheric pressure, which corresponds to a temperature of 89°C. Woollen blankets treated 50 times by this process were undamaged. Vegetative organisms were destroyed but not spores.

The method is suitable for large-scale disinfection of blankets and for disinfecting various other articles which would be damaged at higher temperatures.

The regular disinfection of bedclothes is now being undertaken in many hospitals in order to reduce the risks of cross-infection. In the Bristol Royal Infirmary, the present practice is to disinfect all bedding (including bedcovers and dressing gowns as well as blankets and sheets) once a fortnight, or when the patient leaves hospital.

Non-woollen goods are adequately disinfected by laundering in hot water but woollen blankets present a special problem. While they may be disinfected satisfactorily by laundering with quaternary ammonium compounds (Blowers and Wallace, 1955) or in hot water at a slightly acid *p*H (Dickinson, Wagg, and Fairchild, 1959), pressure of work and lack of space in the laundry of this hospital make it difficult to treat the large number of blankets required (approximately 250 per day). The same problem would exist with non-woollen blankets. For this reason a vacuum formaldehyde process has been used routinely for the past four years (Gillespie and Alder, 1957; Caplan, 1959). Investigation of the temperature in the chamber revealed that the blankets on the outside of the load were raised to temperatures of between 90° and 95°C. Despite this heating, no damage to the blankets was detected and this suggested that woollen blankets might be safely disinfected by steam at temperatures not much below 100°C., if the steam could be made to penetrate through several thicknesses of blanket material. The recent development of high pre-vacuum autoclaves has made this practicable.

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PRINCIPLE

The chamber and its contents are thoroughly evacuated of air so as to allow rapid penetration of steam into the load. Steam is admitted to a pressure of 10 in. Hg below atmospheric pressure, which corresponds to a temperature of 89°C. (Fig. 1). The wall of the chamber is kept warm to prevent heat loss, and condensate is removed by steam traps capable of operating below atmospheric pressure.

METHOD

Most of the experiments were done with a Slater horizontal, jacketed, gravity-displacement autoclave, of 25

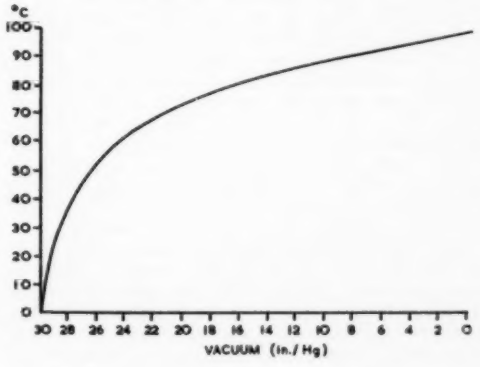

FIG. 1. *Temperatures of saturated steam at low pressures.*

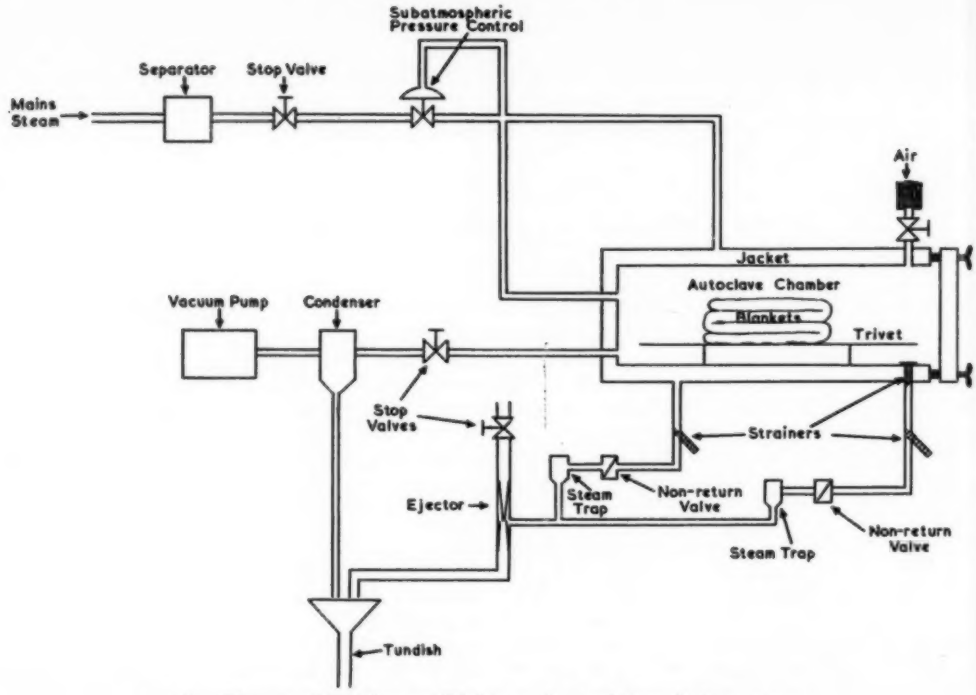

FIG. 2. *Diagram of autoclave modified to work at subatmospheric pressures.*

cubic foot capacity. The autoclave had been in use since 1949, and was recently upgraded to 'high pre-vacuum status' by fitting an Edward's oil-sealed pump and condensing unit, and Drayton automatic controls (Alder and Gillespie, 1959). The piping arrangements are shown in Fig. 2. Two modifications were made so as to allow the autoclave to run at subatmospheric pressures: (1) A control valve fitted at the steam inlet was operated automatically by the pressure in the chamber. The valve could be adjusted to respond to pressures over a wide range, but in the present experiments (except when otherwise stated) it was set to close when the pressure reached minus 10 in. Hg. (2) A water ejector was fitted to the chamber and jacket drains in order to keep the pressure on the outlet side of the balanced pressure steam traps below minus 10 in. Hg. Without this modification the traps could not operate.

The standard process was operated as follows:

Blankets, folded once lengthwise, were stacked inside the chamber. Up to 30 hospital blankets could be accommodated in one load. The closed chamber was then evacuated down to minus 29·25 in. Hg (20 mm. positive pressure), after which steam was admitted to the chamber. The disinfection period was timed from the moment when the temperature in the centre of the blanket pile reached 65°C. A 30-minute period of disinfection was used in most of the experiments. At some time during this period, the chamber pressure reached minus 10 in. Hg and thereafter fluctuated by plus or minus 2 in. Hg as the automatic control valve opened and shut. After the disinfection period, the chamber was evacuated again to a pressure of minus 29 in. Hg. to dry the blankets. Filtered air was then admitted to the chamber, and the blankets removed.

A few experiments were done at higher temperatures.

Records of temperatures at various places inside the chamber and inside the load were obtained by means of thermistors (Alder and Gillespie, 1957) or mercury-in-steel thermometers.

RESULTS

HEAT PENETRATION Many experiments were carried out to record changes in temperature at the centre and at the outside of loads of 25 to 30 blankets.

The temperature in the centre of the load, inside 50 thicknesses of blanket, rose promptly with the pressure but always lagged about 2°C. below the ambient chamber temperature. Figure 3 shows the temperature and pressure recordings in a typical experiment.

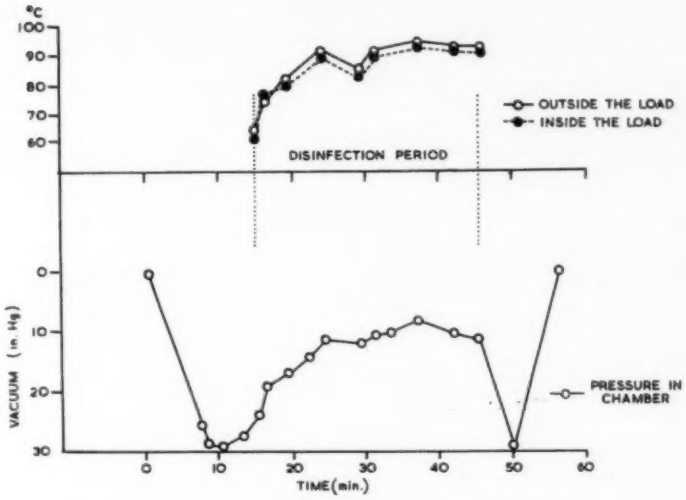

FIG. 3. *Temperatures inside and outside a blanket load, in relation to steam pressure.*

BACTERIOLOGICAL EFFECTS Sweep plate cultures taken from blankets before and after treatment by the method of Williams (cited by Blowers and Wallace, 1955) showed that vegetative organisms were consistently killed but that there was little destruction of sporing organisms.

The bacteriological efficiency of the method was also demonstrated by placing test organisms on dried paper discs enclosed in 2 in. × $\frac{3}{8}$ in. test tubes plugged with cotton wool. The tubes were placed at various depths throughout the load. The test organisms were *Staphylococcus aureus*, *Streptococcus faecalis*, *Eschericia coli*, and *Mycobacterium tuberculosis* (H 37 RV). The test discs, together with unheated controls, were cultured in broth at 37°C. for three days. Discs impregnated with tubercle bacilli were tested for sterility by culture for two weeks in Dubos liquid medium and by guinea-pig inoculation.

With a disinfection period of 30 minutes, the test organisms were killed, except on one occasion when tubercle bacilli and staphylococci survived at the bottom of a load of blankets. When the disinfection period was shortened to 20 minutes or less there were more survivals as shown, in the Table.

EFFECTS ON BLANKETS Since the physico-chemical effect of heat on wool partly depends on *p*H, three identical new woollen blankets were sent to the Wool Industries Research Association where their reactions were adjusted to *p*H 4·0, 7·0, and 9·8 respectively. The blankets were then returned to Bristol and disinfected 50 times by the standard

TABLE

DISINFECTION OF TEST ORGANISMS INSIDE LOADS OF BLANKETS EXPOSED TO STEAM AT SUBATMOSPHERIC PRESSURES

Duration of Disinfection Period (after temperature reached 65°C.)	*Final Temperatures*	*Test Organisms*									
		S. aureus		B. coli		Str. faecalis		M. tuberculosis		B. subtilis	
		Killed	*Survived*	*Killed*	*Survived*	*Killed*	*Survived*	*Killed*	*Survived*	*Killed*	*Survived*
15 minutes	80°-83°C.	10	3	—	—	5	1	—	—	—	—
20 minutes	80°-85°C.	12	1	3	1	—	—	—	—	0	9
30 minutes	86°-90°C.	89	1[1]	3	0	55	0	12	1[1]	—	—
30 minutes	100°C.	14	0	—	—	—	—	—	—	14	0

[1] A pair of tests at the bottom of a pile of blankets in one experiment (See text)

process. Strips of material were removed after 25 and 50 treatments and sent back to the Association, which reported that at the end of 25 treatments there was no noticeable deterioration in the physical and chemical properties of the blanket, and the wearing properties did not appear to be impaired. After 50 treatments the Association again reported that there was virtually no change in the strength of the blankets and changes in the chemical constants were not appreciable.

DISCUSSION

The advantages of the subatmospheric steam method are speed and cheapness. After treatment the blankets are dry. In some recent experiments it has been found possible to disinfect approximately 75 blankets per hour, using a 55 cubic foot autoclave. The method is faster than the vacuum formalin process, and pleasanter for the operator. The experiments reported here were carried out with an autoclave which was also in daily use for the high pre-vacuum steam pressure sterilization of dressings and gloves. But it would probably be undesirable to use the same autoclave routinely for both purposes because of the possibility of a mistake by the autoclave operator. It would be preferable to reserve an autoclave for low-pressure steam work only. Many hospitals nowadays have spare autoclave chambers rendered redundant by the purchase of new high pre-vacuum equipment. An old autoclave could be modified by fitting a suitable pump, condenser, pressure-controlled steam inlet valve, water ejector, temperature recorder, vacuum and pressure gauges, provided only that the chamber is sufficiently free from leaks to allow the achievement of a high vacuum.

In addition to these modifications, it would probably be advantageous to fit a wider inlet pipe and thus obtain a more rapid entry of steam into the chamber than was obtained in the experiments reported here. The failure to sterilize one pair of test organisms, described above, suggested that the safety margin should be increased. This could be done by increasing the rate of entry of steam, or by slightly prolonging the disinfection period.

In routine use, it would neither be practicable nor necessary to take temperature readings from the centre of the blanket load. The disinfection period could be timed from the moment when the temperature in the chamber drain reached 70°C. The adequacy of the disinfection process should be checked periodically by means of control organisms.

The cost of modifying an existing chamber would depend on its capacity, which governs the size of the pump. For a 25 cubic foot autoclave the cost would probably be about £700, for a 50 cubic foot autoclave, about £900. There seems no reason why an automatic control should not be fitted to a subatmospheric steam sterilizer. This would cost about £300 more. If an autoclave were already fitted with high pre-vacuum equipment, the extra fittings for manual control of the subatmospheric steam process would cost about £150.

The subatmospheric steam method should be suitable for the disinfection of other heat-sensitive materials besides blankets, when it is not considered necessary to destroy sporing bacteria. Wooden toys, books, and eiderdown quilts have been treated without damage. It is reasonable to suppose that clothes and mattresses could be disinfected, but this has not yet been investigated.

We wish to thank Mr. J. C. Dickinson for his advice, Mr. J. Barritt for his analysis of the blankets, Mr. K. M. Henfrey for designing the autoclave fittings, and Mr. C. L. Leitch for his advice and generous help with the experiments.

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Nasal abnormality and the carrier rate of *Staphylococcus aureus*

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SYNOPSIS In a group of 178 hospital nurses minor nasal abnormalities were found to be associated with an increased rate of nasal carriage of *Staphylococcus aureus*.

The *Staphylococcus aureus* is found in the anterior nares of 30 to 50% and on the skin in 12 to 25% of healthy adults (Miles, Williams, and Clayton-Cooper, 1944; Williams, Blowers, Garrod, and Shooter, 1960). It is not known why some people carry the organism and others do not, although nasal carriage predisposes to skin carriage as the result of passive transfer. Despite this relationship, in about half the cases of skin carriage the organism is not found in the nose (Miles *et al.*, 1944), neither is there any correlation between the degree of nasal colonization and the intensity and extent of contamination of the skin (Hare and Ridley, 1958).

The incidence of staphylococcal carriage is apparently random and it has been stated that neither congenital anomalies of the upper respiratory tract (Cunliffe, 1949) nor non-staphylococcal disease of the nose (Williams *et al.*, 1960) predisposes to a higher nasal carrier rate. Although this may be true for the staphylococcus, McCartney and Harvey (1928) found that in the case of *Corynebacterium diphtheriae* nasal abnormality favoured the development of the carrier state. Subtle constitutional differences of the skin have been suggested as the reason for staphylococcal carriage on the integument (Tulloch, 1954) but this has not been demonstrated experimentally.

The paper records an attempt to determine some of the factors that may predispose to staphylococcal colonization of the nose and the skin in individuals without any obvious staphylococcal infection.

METHODS

One hundred and seventy-eight nurses, some of whom were in the preliminary training school and had not been in contact with a hospital environment, formed the group for study.

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CLINICAL ASSESSMENT An E.N.T. and dermatological history of each subject and her immediate family was taken. An aural surgeon examined the nose and throat in each individual and noted any clinically significant abnormality, and a dermatologist placed the subject into one of four skin types, namely, normal, dry, moist, or greasy. The presence of minor skin sepsis was also noted. A special note was made of recent treatment with chemotherapeutic agents.

BACTERIOLOGICAL SAMPLING Three swabs moistened with nutrient broth were taken from the following sites: the palmar surface of the left wrist, the nasal vestibule, and the mucosa immediately above the anterior end of the inferior turbinate. Under direct vision and with instrumentation an aural surgeon swabbed the inferior turbinate. A fourth swab was taken from the tonsillar area in the last 66 nurses.

The swabs were plated on to nutrient agar without delay. After 48 hours' incubation the plates were examined for coagulase-positive staphylococci. The phage and sensitivity patterns of these were determined. To determine sensitivity, Sentest (Evans) tablets were used on nutrient agar plates previously seeded with 1 ml. of a 1 in 100 dilution of an 18-hour broth culture.

RESULTS

The incidence of skin carriage was so low (7·25%) that any correlation with clinical skin types was unjustified.

The overall incidence of nasal carriage was 38% (68 out of 178). With one exception all were vestibular carriers, and in almost half (17%) carriage extended far enough to involve the mucosa above the inferior turbinate.

The staphylococci isolated were distributed amongst all phage groups (Table I). Table I also records their susceptibility to antibiotics. It can be seen that the populations studied, with respect to the character of the staphylococci present, were

TABLE I

CHARACTERISTICS OF STAPHYLOCOCCI ISOLATED FROM THE NOSES OF 178 NURSES

Sensitivity Patterns of Strains for				No. of Ward Nurses Yielding Strains of Staphylococci					No. of Preliminary Training School Nurses Yielding Strains of Staphylococci				
Penicillin	Streptomycin	Tetracycline	Erythromycin	This Sensitivity Pattern	Phage Group I	II	III	Others	This Sensitivity Pattern	Phage Group I	II	III	Others
S[1]	S	S	S	3	1	2	0	0	14	6	4	2	2
R[1]	S	S	S	35	19	1	3	12	6	3	2	1	0
R	R	S	S	1	0	0	0	1	0	0	0	0	0
R	R	R	S	4	0	0	3	1	0	0	0	0	0
R	R	R	R	5	0	0	5	0	0	0	0	0	0
Total No. of Nurses													
Yielding staphylococcus strains				48	20	3	11	14	20	9	6	3	2
Examined				107					71				
Percentage of nurses positive				45	19	2·7	10·3	13	28	12·6	8·5	4·2	2·7

[1]S = sensitive; R[1] = resistant

No strain was found to be resistant to either chloramphenicol or novobiocin.

TABLE II

PHAGE GROUP DISTRIBUTION OF STAPHYLOCOCCI ISOLATED FROM NURSES WITH NORMAL AND ABNORMAL NOSES

Nurses with	Where Stationed	No. Carrying Staphylococci of Phage Group				No. of Non-carriers	Total
		I	II	III	Others		
Abnormal noses	Ward	4 } 4	0 } 0	3 } 5	4 } 4	4 } 7	20
	Preliminary training school	0	0	2	0	3	
Normal noses	Ward	16 } 25	3 } 9	8 } 9	10 } 12	55 } 103	158
	Preliminary training school	9	6	1	2	48	
Total		29	9	14	16	110	178

comparable to those of previous workers, *e.g.*, Barber and Burston (1955).

A clinically significant minor nasal abnormality, *i.e.*, a deviated septum or damaged turbinate, was found in 20 individuals and of these 13 (65%) carried a coagulase-positive staphylococcus in the nose. In the remainder, those having normal noses, 55 (29%) were nasal carriers. The incidence of staphylococcal carriage in the group with nasal anomalies was significantly higher than that in the group with normal noses ($\chi^2 = 6{\cdot}88$, $n = 0{\cdot}01 > p > 0{\cdot}001$).

This relationship was further strengthened by the finding that staphylococcal carriage in the throat was almost exclusively confined to those individuals who showed some abnormality of the tonsil (Campbell, 1948). Thus, of 66 subjects examined, six had tonsillar disease and all of these carried *Staphylococcus aureus* in the throat. On the other hand, of the 60 subjects with normal throats only one carried a *Staphylococcus aureus* in this area. These findings are analogous to those of Hartley and Martin (1920), who found that the persistence of carriage of *Corynebacterium diphtheriae* was apparently related to the presence of diseased tonsils. In the present investigation no relationship was observed between mucosal carriage and tonsillar carriage (Dingle and Plummer, and others, 1949).

Nasal abnormality does not favour colonization of the nose by staphylococci of any particular phage group (Table II), nor does it predispose to involvement of the upper reaches of the nose (Table III).

TABLE III

EXTENT OF COLONIZATION OF NORMAL AND ABNORMAL NOSES BY STAPHYLOCOCCI

Nurses with	No. Carrying Staphylococci on/in			Total
	Mucosa Alone	Mucosa and Vestibule	Vestibule Alone	
Abnormal noses	0	6	7	13
Normal noses	1	25	29	55
Total	1	31	36	68

DISCUSSION

It has been stated that nothing is gained in the estimation of staphylococcal carrier rates b

swabbing the upper reaches of the nose, and indeed that misleadingly low carrier rates may be obtained by so doing (Williams *et al.*, 1960). Stratford *et al.* (1960), however, isolated *Staphylococcus aureus* from mucosal swabs in 51% of a group of 103 patients but from vestibular swabs taken from the same group in only 34%, a result that throws some doubt on the obvious criticism of mucosal sampling that the organisms isolated might be contaminants derived from the vestibule. The technique used by Stratford, Rubbo, Christie, and Dixson (1960) is not detailed; in our tests the vestibule was opened out with a Thudichum speculum and samples taken from the clearly visualized inferior turbinate region. In our experience the mucosa is rarely involved alone.

In view of the findings of Cunliffe (1949), who found no increase in the staphylococcal carriage rate of children with gross abnormality of the nasopharynx, our finding that there is a higher rate of nasal carriage in nurses with minor nasal abnormalities might appear surprising, However, this result for the staphylococcus is in accord with that of McCartney and Harvey (1928) for *Corynebacterium diphtheriae*, who found that 72% of diphtheria carriers showed nasal abnormalities, in contrast to patients who had recovered clinically from diphtheria and were also bacteriologically free in whom the abnormality rate was only 7%. Moreover in the discussion on the epidemiology of scarlet fever, Wilson and Miles (1955) suggest that abnormalities of the upper respiratory tract prolong carriage of *Streptococcus pyogenes*.

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A contribution to the technique of intracutaneous testing with toxoplasmin

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SYNOPSIS The preparation and application of toxoplasmin for intradermal testing are described. The persistences of sensibility to toxoplasmin and the specificity of the test are discussed.

A great deal of attention has been directed to acute toxoplasmosis, congenital and acquired, and much less to chronic and latent infections. These cannot readily be investigated by the direct methods of demonstrating the parasite in histological sections or by isolating it by animal inoculation. Recourse has to be had to indirect, antigen-antibody reactions. In addition to the Sabin-Feldman dye test, the complement-fixation test, and the haemagglutination test, we use the intradermal test with toxoplasmin. Over the past six years we have performed this test on 15,000 persons. This paper describes the preparation and use of the antigen. We intend to report in a subsequent paper the results obtained of tests performed on normal and other groups of the population.

PREPARATION OF ANTIGEN FOR THE INTRADERMAL TEST

The main modifications on the original method of Frenkel (1948) are that we do not use ultra-violet light, because it may destroy proteins, and we do not add heparin.

Fifty to sixty mice of about 20 g. weight, from stocks known to be free from salmonellosis and ectromelia, were inoculated intraperitoneally with 0·1 ml. of a 1 : 10 to 1 : 20 dilution in saline of fresh mouse peritoneal exudate containing large numbers of toxoplasma (Bk strain). Four days later the mice were killed, and peritoneal exudate aspirated with aseptic precautions. Each mouse yielded from 0·2 to 1·0 ml. The exudates from groups of 10 mice were pooled in sterile tubes. Tests for bacterial sterility were set up. Then the material was centrifuged at 3,000 r.p.m. for 30 minutes and the supernatant fluid discarded. The deposit, consisting of toxoplasma and white blood corpuscles, was stored at −20°C. until the results of the sterility test were known. Then it was added in about 3 g. amounts to 3 ml. of normal saline containing 0·25% of phenol. The mixture was homogenized in a Potter-Elvehjem all-glass tissue grinder for five minutes, after which it was thrice frozen to −70°C and thawed. It was then diluted 1 : 10 with phenol saline and stored in a 4°C. refrigerator for three weeks, during which time it was shaken daily. After this it was centrifuged, and the supernatant fluid removed. This was thrice frozen and thawed and then centrifuged to get rid of particulate matter, which would otherwise precipitate and have no antigenic value. The antigen thus prepared is stored in the refrigerator and diluted from time to time as required.

The antigen is tested for sterility and absence of toxicity or living toxoplasma by the State Institute for the Control of Drugs. The latter test is done by inoculating mice in three successive passages. Up to the present no toxic reactions have been observed in mice inoculated with the dilutions used for man.

TITRATION OF ANTIGEN The dilution at which the antigen is used is the highest which gives a characteristic, well-defined reaction in an allergic person. Much depends on how the antigen is made, particularly its homogenization. Early batches could not be diluted beyond 1 : 200 to 1 : 500. Current batches will stand dilution of 1 : 1,000 to 1 : 2,000 giving identical intensity of cutaneous reaction. Each fresh batch is tested in parallel with the previous batch on 10 to 15 negatively-reacting and on the same number of positively-reacting persons.

Tests on 104 persons have shown that the cell-free portion of toxoplasma containing mouse peritoneal exudate has only weak antigenicity in dilutions of 1 : 5 to 1 : 100.

TECHNIQUE OF THE INTRADERMAL TEST

We inoculate the control solution, phenol saline, into the cleansed skin above the insertion of the deltoid muscle and the antigen below the insertion. These sites have been chosen as being as sensitive as and less painful than the anterior aspect of the forearm. Instead of judging the amount of inoculum by the quantity expelled from the syringe, which is subject to loss through leakage, we do so by the size of wheal produced, which should measur

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8 mm. in diameter. Usually this is produced by 0·07 to 0·08 ml. of antigen.

In a very large number of tests we have had no serious reactions. Rarely there are general symptoms such as evening fever, swelling of lymph glands, especially in the axillae and groins, chills, and vomiting. Occasionally necrosis has developed at the site of infection but healed within 10 days. In one patient a psoriasis-like eruption developed. Care should be taken in the inoculation of patients with ocular inflammations, particularly chorio-retinitis, for they may have an allergic exacerbation, which can, however, be suppressed by cortisone. We would emphasize that toxoplasmin is thermostable. Syringes used for controls should be kept separately and carefully rinsed.

READING OF TEST

The test is read after 24 hours and again after 48 hours. Rarely a person negative at 24 hours develops a reaction after 48 hours. In such cases the maximum reaction does not appear till 72 hours. All negatives should be examined again after 72 hours. The size of erythema and induration is measured in millimetres in two cross diameters and scored as in Table I. We consider reactions read as + of no significance and a ++ reaction of doubtful significance.

TABLE I

EVALUATION OF THE TOXOPLASMIN TEST

		Time of Reading		*Result*
		After 24 Hours	*After 48 Hours*	
Erythema[1]	Size	20-40 × 15-20	40-80 × 30-60	
	Intensity	++++	++++	Strong
Induration[1]	Size	15-25 × 10-20	15-35 × 10-30	Positive
	Intensity	+++	+++	++++
Vesicle	Size	10 × 10	15 × 15	
Erythema	Size	15-20 × 10-15	20-30 × 15-30	
	Intensity	+++	++++	Positive
Induration	Size	8-12 × 6-10	10-15 × 8-10	+++
	Intensity	++	+++	
Erythema	Size	12-15 × 8-12	8-12 × 8-10	
	Intensity	++	++	Weak
Induration	Size	8-10 × 6-8	6-10 × 6-8	Positive
	Intensity	++	+	++
Erythema	Size	4-8 × 3-6	2-4 × 2-3	
	Intensity	+	+	Negative
Induration	Size	3-6 × 2-4	0	
	Intensity	+	0	+ − 0

[1] Size of erythema and induration in mm.

DEVELOPMENT AND PERSISTENCE OF SENSITIVITY TO TOXOPLASMIN

We re-examined three groups at intervals of one to four years with the toxoplasmin test.

1 Of 108 persons negative at the first test, 165 (91·7%) remained negative, five (2·8%) became weakly positive, and 10 (5·5%) clearly positive. Two of the latter worked with laboratory animals, which had not, however, been intentionally infected with toxoplasma. One of these, as soon as her illness was clinically obvious, already had a dye test titre of 1 : 16,000, a complement-fixation titre of 1 : 160, and a strongly positive intradermal test. In another three cases the complement-fixation test became positive before the toxoplasmin skin test. In yet another case, not included in this series, a mother immediately after the delivery of a foetus proved to have toxoplasmosis, gave a negative complement-fixation test and skin test. Three months later a complement-fixation test was positive but not till eight months after delivery did a skin test become positive.

2 Of 130 persons weakly positive (++) at the first test, 53 (40·8%) gave the same reaction, 32 (24·6%) gave a negative reaction, and 45 (34·6%) gave a clearly positive reaction.

We believe that those who were first ++ and then negative had shown a non-specific reaction.

3 Of 363 persons strongly positive (+++ or ++++) at the first test, 208 (57·3%) remained the same, 44 (12·1%) fell from ++++ to +++, 52 (14·3%) fell from +++ to ++, and 59 (16·3%) rose from +++ to ++++. We have not observed an original +++ or ++++ become negative.

Persistence of skin sensitivity has been proved in one instance for 37 years (the mother of the first case ever described, by Janků in 1923); in two for nine years (in one of these instances the infected daughter survives and is also positive), and in another for five years.

SPECIFICITY OF THE TOXOPLASMIN INTRADERMAL TEST

It might be objected that the reactions were due to non-specific material. To meet this we prepared and tested toxoplasmins from (*a*) infected mouse peritoneal exudate, (*b*) infected chick-embryo chorio-allantoic membrane, (*c*) infected tissue culture and at the same time, as controls, we tested similar but non-infected preparations.

Chick-embryo toxoplasmin was prepared by inoculating the chorio-allantoic membranes of 10-11-day-old embryos with 0·1 to 0·2 ml. of a 10% suspension of infected mouse peritoneal exudate or of infected chorio-allantoic membrane. Four days later the whole egg membranes, or those parts that showed plaques due to toxoplasma growth, were removed, tested for bacteriological sterility, and kept for several days at −20°C. They were then washed in saline, finely chopped, homogenized in a M.S.E. homogenizer, three times frozen (−70°C.) and thawed (37°C.), and again homogenized. Phenol saline was then added; the subsequent procedure was as for mouse peritoneal exudate toxoplasmin. As the chorio-allantoic membrane antigen contained relatively less toxoplasma material than the mouse peritoneal exudate antigen it was used in lower dilution (1 : 150 to 1 : 200). Table II shows the results of comparative tests with the

TABLE II

COMPARISON OF INTENSITY OF REACTIONS WITH MOUSE PERITONEAL EXUDATE TOXOPLASMIN AND CHICK EMBRYO CHORIO-ALLANTOIC MEMBRANE TOXOPLASMIN ON 390 PERSONS

		Egg Toxoplasmin				
		0	++	+++	++++	*Total*
Mouse toxoplasmin	0	*204*	—	—	—	204
	++	—	*24*	16	3	43
	+++	—	17	*72*	9	98
	++++	—	2	15	*28*	45
	Total	204	43	103	40	390

TABLE III

COMPARISON OF INTENSITY OF REACTIONS OF MOUSE TOXOPLASMIN AND TISSUE CULTURE TOXOPLASMIN IN 417 PERSONS

		Tissue Culture Toxoplasmin					
		0	+	++	+++	++++	*Total*
Mouse antigen	0	*231*	—	—	—	—	231
	+	5	*1*	—	—	—	6
	++	2	8	*18*	7	1	36
	+++	—	1	31	*37*	19	88
	++++	—	—	3	24	*29*	56
	Total	238	10	52	68	49	417

mouse peritoneal exudate antigen and the egg antigen (the latter at a dilution of 1 : 200). The results show agreement in 328 of the 390 tests.

Tissue culture toxoplasmin was prepared by infecting 6-day-old trypsinized monkey kidney cultures (in big Roux jars) with mouse peritoneal exudate containing toxoplasma. After seven days of culture the toxoplasmas were removed from the medium by centrifugation and toxoplasmin prepared as from mouse exudate. The potency of the tissue culture antigen was lower than that of the mouse antigen. It was used in dilutions of 1 : 50 to 1 : 300.

Table III shows the results of comparative tests with the mouse antigen and the tissue culture antigen. Complete agreement was found in 316 persons (75·8%). A really significant difference was found in one case only (+++ with mouse antigen, + with tissue culture antigen). The other differences do not exceed the variations in intensity of reaction to be expected in such a test, for it is impossible to inoculate intradermally absolutely identical amounts of antigen.

The control preparations used and the results obtained with these were as follows:—

1 Sterile ascitic fluid artificially produced in healthy mice was used to test 32 persons. None reacted. 2 Extract of mouse spleen diluted 1 : 500 was used to test 560 persons. Only 11 reacted and they gave no more than + insignificant reactions. 3 Chorio-allantoic membrane of 14-15-day-old chick embryos diluted 1 : 200 was used to test 326 persons. None reacted. 4 Non-infected tissue culture was used to test 326 persons. None reacted. 5 Phenol saline was used to test 12,280 persons and two gave a + reaction.

APPLICATIONS OF THE TOXOPLASMIN TEST

Since the toxoplasmin test is easy to perform and sensitization with positive reaction persists for long periods, it is well suited to estimating the amount of toxoplasma infection there is, or has been, in a community.

It is also of value in assessing the part played by toxoplasmosis in the production of various diseases, congenital and acquired. Thus it can be used in the investigation of the aetiology of congenital developmental defects—mongolism, oligophrenia, and so on. When this is done it is important to test not only the children but also their mothers. The evidence obtained is statistical, hence it is necessary (1) to test a homogeneous control group of approximately the same age, from the same locality, similarly employed; (2) to obtain a careful and detailed clinical diagnosis on the individual members of the test group, *e.g.*, to differentiate granulomatous from other forms of uveitis (toxoplasmosis has thus been found to play a part in the former but not in the latter); to exclude individuals whose illness is shown to be due to another cause; to have sufficient numbers for statistical analysis.

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Antistreptolysin levels in normal infants and young children

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SYNOPSIS A modified method of estimating the antistreptolysin O titre (A.S.O.) is described. The A.S.O. of 279 sera from normal children under 5 years of age is recorded, together with that of 40 children over 5 years of age. The results show a range much lower than that found in adults and older children and are discussed with respect to estimations made in children with proven infections. The significance of these findings is discussed.

Of the serological tests used in the diagnosis of streptococcal infections, the estimation of the A.S.O. has emerged as the most consistent and reliable. First introduced by Todd (1932a and b) and modified by other workers (Hodge and Swift, 1933; Rantz and Randall, 1945) the test is a routine procedure to aid in the diagnosis of rheumatic fever, nephritis, and other post-streptococcal conditions. In adults the upper limit of normal is taken by Rantz (1955) and Lawy (1960) as 200 Todd units/ml., while Wood and McCarty (1954) and Hollinger (1953) consider that 250 units/ml. should be the upper limit. Studying a series of patients suffering from rheumatic fever, Wood and McCarty (1954) demonstrated that 80 to 90% of patients showed significantly raised A.S.O. levels. Stollerman, Lewis, Schultz, and Taranta (1956), in a series of 20 similar cases, claimed that 19 showed titres which are considered significant. McCarty (1952a) asserted that significant levels are found in 70 to 80% of all cases of proven streptococcal infection. In adults and older children, the value of the test is considerable.

In children under 5 years of age, the test is considered to be of limited value. McCarty (1952b) included in a series of rheumatic fever cases two children under 5 years of age. Neither showed at any time an A.S.O. of more than 25 units/ml. Rantz, Maroney, and Di Caprio (1951) investigated 52 cases of proven streptococcal infection in young children and divided them into three categories, depending on the antibody response and the rate of fall in titre. In Rantz's first group, in which the average age was 14 months, a maximum titre of 12 units/ml. was found in six of 13 children, the others showing no response. In the second group, in which the average age was 36·7 months, six of 18 showed peak titres of 50 units/ml. while the mean peak titre was 235 units/ml. The levels, however, fell to 12 units/ml. in a mean time of 40·5 days for the six showing a small response and a maximum of 130 days for the other 12. In the third group, in which the mean age was 57·9 months, the titres found and the rate of fall was comparable to the pattern found in adults and older children. Of 18 normal children between the ages of 1½ and 3 years, Rantz found levels of less than 12 units in 17.

During the first weeks of life high levels were found. Lippard and Wheeler (1936) showed that this was due to passively acquired maternal antibody. This was also noted by Wilson, Wheeler, and Leask (1934). It appears that the normal levels and titres found in cases of proven streptococcal infection are of a very different order in children under 5 years of age as compared with older children and adults. Estimations carried out on children of this age group cannot be interpreted in terms of adult normal and abnormal values.

The present investigation was carried out in order to find what A.S.O. levels could be expected in normal children under 5 years of age, and to serve as a base line for the interpretation of results found in clinical cases of infection.

MATERIALS

From sera submitted to the laboratory in excess of the laboratory's requirements for routine tests in a variety of conditions, 319 were collected and stored at −20°C. The sera were in the main from infants examined before

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adoption and clean surgical cases admitted for treatment (burns, cleft palate, hare lip, and accident). Sera from children with renal disorders, leukaemia, or with a history of acute or post-respiratory tract infections or blood transfusions were not included.

The method of estimation of A.S.O. levels has been modified by one of us. Details are given in the Appendix (C.W.P.).

RESULTS

Table I shows the number of sera giving the same A.S.O. for the corresponding age group. No levels of 200 units/ml. or more were found in 319 sera tested, and 152 showed no detectable A.S.O. at a dilution of 1/8 (Table II shows the summarized results). In infants under 2 months of age the A.S.O.s are of a higher order than in any other age group under 5 years of age. Only 11 out of 85 (13%) show no detectable level and 51/85 (60%) showed levels between 32 and 192 units/ml. In contrast, no detectable levels were found in 84% and 86% respectively in infants aged 2 to 12 and 13 to 24 months. Only three of 126 sera from children between 2 months and 2 years of age showed titres of 32 units/ml. or more. Between the ages of 2 years and 5 years, however, only 25 of 68 (36·8%) gave undetectable titres and 31 (45·6%) gave titres of 32 units or more. These levels represent the levels that exist in a cross section of presumably normal children of these age groups and are much lower than in older children or adults. Forty sera from children 5 years to 7 years of age are included for comparison. Detectable levels may be due to past subclinical or undiagnosed infections by β-haemolytic streptococci.

DISCUSSION

It is generally agreed that in children under 2 months of age the antistreptolysin 'O' is passively acquired from the mother. The levels fall quickly and disappear at about 2 months of age, as can be seen from Table I. In interpreting results obtained in these young babies this factor must be taken into account, as this fall could mask any response which might occur as a result of streptococcal infection. In fact, it is not yet known if any response does occur in the presence of maternal antibody.

Between the ages of 2 months and 2 years detectable antibody was found in only 19 of 126 sera. Further studies of this age group are necessary to find whether these titres are significant as evidence of past infection by comparing them with titres from children with proven β haemolytic streptococcal infections. Rantz *et al.* (1951) found that of 13 children (average age 14·3 months) with proven infections, six gave titres of 12 units/ml., while of our normal series only 11 out of 126 have titres of

TABLE I

NUMBER OF SERUM SPECIMENS GIVING SIMILAR A.S.O. FOR THE VARIOUS AGE GROUPS

A.S.O. (Todd units/ml.)	*Age (Months)*																	
	0-2	*3-4*	*5-6*	*7-8*	*9-10*	*10-12*	*13-14*	*15-16*	*17-18*	*19-20*	*21-22*	*23-24*	*25-30*	*31-36*	*37-42*	*43-48*	*49-54*	*55-60*	*>60*
192->	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	—	1
128-191	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	7
96-127	7	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1	3	1	5
64- 95	12	—	—	—	—	—	—	—	—	—	—	1	1	1	2	3	1	1	9
48- 63	17	—	—	—	—	—	—	—	—	—	—	—	—	2	2	1	—	2	1
32- 47	13	1	—	1	—	—	—	—	—	—	—	—	1	1	1	1	1	1	3
24- 31	10	—	—	—	—	—	—	—	—	—	—	1	3	—	—	—	—	1	1
16- 23	10	—	—	2	1	1	1	—	—	—	—	—	2	—	1	—	—	1	4
12- 15	1	1	—	—	—	—	—	—	—	—	—	1	—	—	1	—	—	—	—
8- 11	2	1	2	2	—	—	1	—	2	—	—	—	1	—	1	—	—	1	—
<8	11	13	15	12	13	11	10	9	6	3	3	12	10	7	3	2	2	1	9

TABLE II

SUMMARIZED RESULTS OF A.S.O. LEVELS FOR VARYING AGE GROUPS WITH CORRESPONDING PERCENTAGES OF TOTAL NUMBERS

A.S.O. (Todd units/ml.)	*Age (Months)*				*Total*
	0-2	*3-24*	*25-60*	*>60*	
>32	51 (60%)	3 (2·3%)	31 (45·6%)	26 (65%)	111
8-31	23 (27%)	16 (12·7%)	12 (17·6%)	5 (12·5%)	56
< 8	11 (13%)	107 (85%)	25 (36·8%)	9 (22·5%)	152
Total	85	126	68	40	319

12 units or more. The analysis of Rantz's cases was based on the type of response rather than on age.

Between the ages of 2 years and 5 years of age (Table I) the number of sera showing no detectable A.S.O. decreased and the number of sera showing measurable levels tended to increase with age. This is as would be expected after repeated contact with β haemolytic streptococci. Rantz *et al.* (1951) found that in cases of proven streptococcal infecton two groups of children with average ages of 36·5 months and 57·9 months gave average peak titres of 235 units/ml. and 495 units/ml. respectively. In our normal children, however, only nine of 68 sera showed titres of over 100 units/ml.

Analysis according to age is not the only method of approach to this problem, for it has been suggested that the response to infection, either clinical or subclinical, is probably dependant on whether response has been initiated by previous contact with the antigenic stimulus and only a detailed history would give the position of an individual patient with respect to past infection. Rantz *et al.* (1951) and Rantz (1955) have shown that initiation by proven infection allows for a more active response when a child is reinfected.

The significance, therefore, of A.S.O. levels in children in the younger age groups should be considered with respect to normal levels in a corresponding age group and not against normal levels established in older persons. In this respect the work of Holborow and Isdale (1956) on the 'sensitization' concept of Powers and Boisvert (1944) and Rantz *et al.* (1951) should be reconsidered.

APPENDIX

MATERIALS The following were used:—

1 Haemolysin 0 is the standard haemolysin 0 (Difco Laboratories).

2 Isotonic buffer solution and red cell suspension (Rantz and Randall, 1945).

3 Perspex haemagglutination trays (Prescott Ltd.).

4 The pipette is the interchangeable, automatic Everett pipette (Turner & Sons Ltd.) of 0·2 ml. delivery volume.

METHOD By placing two or more trays side by side the number of dilutions tested can be made as large as required. For the purposes of this investigation 12 dilutions were tested. A single pipette was used throughout.

1 To a horizontal row of wells, 1 to 12, one volume of buffer was added to all the wells except well 1.

2 From a serum dilution of 1 : 4, 1 vol. was placed in well 1, 2 vol. in well 2, and 1 vol. in well 3. The pipette was flushed out twice with clean buffer solution, discarding each washing.

3 The contents of well 3 were mixed with the automatic pipette and 1 vol. transferred to well 5. The pipette was flushed out once with clean buffer solution, discarding the washing, a procedure repeated after every subsequent transfer of volumes unless otherwise stated.

4 The contents of well 5 were mixed with the automatic pipette and 1 vol. transferred to well 7. This process was repeated for all the odd numbered wells in the series, a volume from well 11 being discarded. The pipette was not flushed out after well 11.

5 The contents of well 2 were mixed, 1 vol. was discarded and 1 vol. transferred to well 4.

6 The contents of well 4 were mixed and one volume transferred to well 6. This is continued for all the even numbered wells, discarding 1 vol. from well 12.

7 After the dilution series was completed the pipette was flushed out 10 times, discarding each washing, and 1 vol. of buffer was placed in every well. The resulting series of dilutions was 1 : 8, 1 : 12, 1 : 16, 1 : 24, 1 : 32, etc. to 1 : 384.

8 The trays were left at 37°C. for 30 min.

9 One volume of standard haemolysin 0 was added to each well, and the trays further incubated at 37°C. for 15 min.

10 One volume of the 5% red cell suspension was added to each well and the trays incubated for 45 min. at 37°C.

11 The trays were then placed at +4°C. overnight and then read against a white background. Titres were given as the highest dilution of sera giving complete neutralization of the haemolysin suspension, *i.e.*, showing no trace of lysis.

If the stock serum dilution of 1 : 50 is taken and the same technique used the dilution series is 1 : 100; 1 : 150; 1 : 200; 1 : 300, etc., and this method was used for estimations carried out in routine laboratory investigations.

One hundred and fourteen sera with titres between 12 units/ml. and 800 units/ml. were carried out in parallel with the method of Rantz and Randall (1945). The results are comparable and results using our technique are expressed in Todd units/ml. Once familiar with using an automatic pipette, the technique is both time saving and easier to prepare. Smaller quantities of reagents are required and results are easily interpreted.

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Fatal septicaemia due to *Salmonella cholerae suis*

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SYNOPSIS A fatal case of *Salmonella cholerae suis* septicaemia in a patient with mitral stenosis is described. At necropsy infection had become localized in an auricular thrombus.

Salmonella cholerae suis was first isolated by Salmon and Smith who named it the American hog cholera bacillus and considered it to be the cause of hog cholera. Since then, however, it has been established that hog cholera is of viral origin with *S. cholerae suis* as a secondary invader (Wilson and Miles, 1955). Human infection with this organism was first reported by Longcope (1902) who described two cases of a typhoid-like infection due to a 'paracolon' bacillus shown subsequently by Tenbroeck, Li, and Yü (1931) to belong to the hog cholera group. Since then numerous case reports have appeared in the American literature (Saphra and Winter, 1957); in Great Britain only 10 full case reports have appeared (Table I). In all cases the clinical pattern of the infection falls into one or more of three groups: (1) A typhoid-like state or septicaemia, (2) focal manifestations outside the alimentary tract, and (3) a gastro-enteritis. The incidence of the infection is low. Of 7,779 salmonella infections of all types identified at the New York Salmonella Center between 1939 and 1955, only 359 (4·6%) were due to *S. cholerae suis* (Saphra and Winter, 1957). Out of a total of 16,260 salmonella infections notified in the Public Health Laboratory Service weekly summaries for England and Wales in 1958 and 1959, only 28 (0·17%) were due to *S. cholerae suis;* these 28 cases occurred as isolated instances and were scattered throughout the country. No case was reported in the Manchester area during these two years. The following case of *S. cholerae suis* septicaemia is reported in view of its rarity, and of the unusual site at which the infection became localized.

CASE REPORT

A woman, aged 51, was admitted to the Manchester Royal Infirmary, under the care of Dr. William Brockbank, on 25 November 1959. In 1949, she was noted to have mitral stenosis and auricular fibrillation and gave a previous history of rheumatic fever at the age of 20. For 10 years she had suffered breathlessness on exertion and for five years orthopnoea and occasional oedema.

On 10 November 1959, after an attack of shivering, she complained of an ache in both loins associated with frequency, dysuria, and fever. A catheter specimen of urine showed no pus cells but a scanty growth of *E. coli* was obtained on culture; this mild infection was treated successfully with sulphafurazole. On 14 November she

TABLE I

CASES OF *Salmonella cholerae suis* INFECTION RECORDED IN GREAT BRITAIN

Author	*Sex*	*Age*	*Sites of Recovery of Organism*	
			During Life	*At Necropsy*
Nabarro *et al.* (1929)	F	8 mth.	Blood, joint fluid	(Alive)
	M	20 yr.	Blood	Heart blood, spleen
Boycott and McNee (1936)	F	35 yr.	Blood	Auricular thrombus, meninges, spleen, gall bladder
Herring and Nicholson (1939)	F	33 yr.	Fallopian tube	(Alive)
Guthrie (1941)	M	7 mth.	—	Periarticular abscess, both middle ears
Schwabacher *et al.* (1943)	F	40 yr.	Blood	Heart blood, spleen, liver
	M	18 yr.	Blood, urine	Spleen, lungs
Laylee (1957)	M	53 yr.	Blood, urine	(Alive)
	F	23 yr.	Pleural cavity	(Alive)
Horler and Ismay (1960)	M	54 yr.	Blood	Para-aortic lymph nodes
Bailey *et al.* (1961)	F	51 yr.	Blood, faeces	Auricular thrombus

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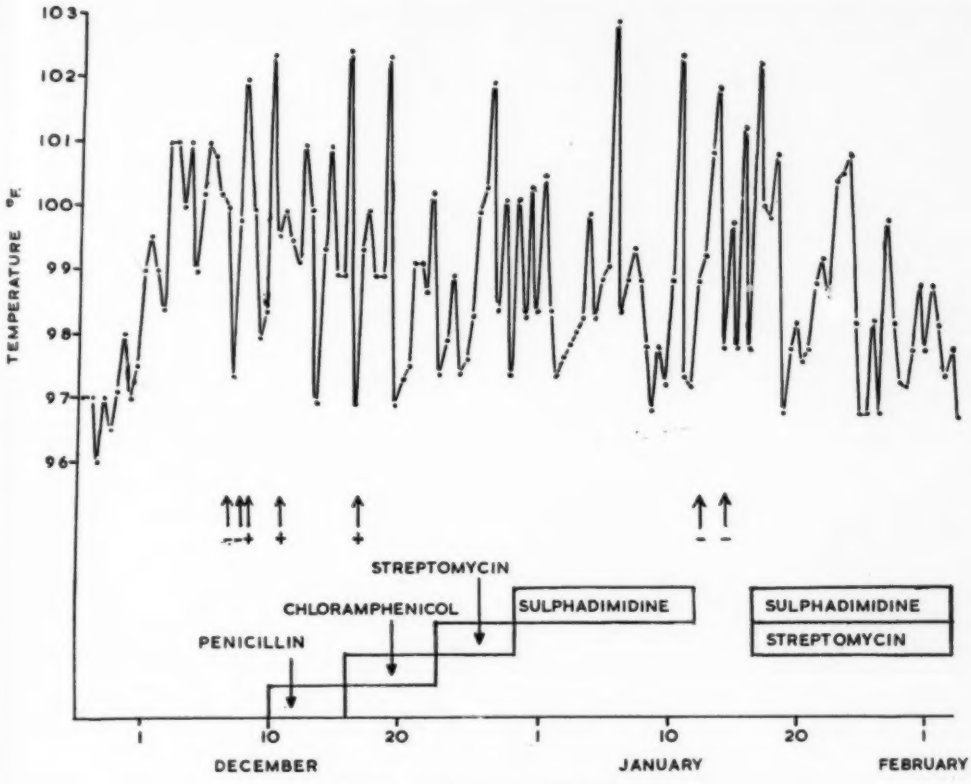

FIG. 1. *Chart of temperature and treatment.*

developed diarrhoea, vomiting, and abdominal colic, and these symptoms persisted for four days. She remained weak with a poor appetite and increasing thirst and because of deterioration in her condition she was admitted to hospital.

On examination she was stuporose with uraemic foetor but afebrile. The heart was enlarged, there were mitral diastolic and systolic murmurs and controlled auricular fibrillation. There was no cardiac failure. The spleen was not palpable. Her urine contained no protein or sugar.

INVESTIGATIONS Haemoglobin was 16·3 g. per 100 ml. (110%); white blood cells 12,400 per c.mm. (80% neutrophils); the erythrocyte sedimentation rate 6 mm. in the first hour (Wintrobe); blood urea 188 mg. per 100 ml. Serum electrolyte levels were sodium 122 mEq./l., potassium 3·8 mEq./l., chloride 70 mEq./l., and plasma bicarbonate 26 mEq./l.

PROGRESS AND TREATMENT Sodium and potassium supplements were given. By 3 December the serum sodium level had risen to 133 mEq./l. and the blood urea had dropped to 33 mg. per 100 ml. The patient felt better and had become alert and cheerful. On 1 December the temperature rose above normal for the first time and thereafter she ran a swinging temperature ranging between 97 and 103°F. (Fig. 1). Diarrhoea did not recur and she did not at any time show any embolic phenomena. The white cell count on 4 December was 6,200; this rose to 17,400 on 7 December but thereafter fell to between 6,000 and 9,000 with isolated rises to 15,000 on 9 and 29 January 1960 and on 3 February 1960.

Blood cultures on 8, 11, and 17 December gave a growth of *S. cholerae suis*, var. *kunzendorf*, after nine days' incubation. Two previous blood cultures on 7 and 8 December, and two blood cultures taken after 17 December were negative. Serum collected on 10 December gave positive agglutination with composite Salmonella H to a titre of 1/80 but was otherwise negative. On 17 December agglutination was obtained with composite Salmonella H to a titre of 1/2,560 and with *S. paratyphi C* 0 to a titre of 1/640. The organism was isolated from faeces sent for examination on 7 December but was not present on 14 December or on subsequent

examinations. Specimens of urine remained sterile after the initial mild infection with *E. coli* had responded to treatment.

Treatment began on 10 December with successive courses of penicillin, chloramphenicol, streptomycin, sulphadimidine, and then sulphadimidine with streptomycin. The fever persisted and her condition gradually deteriorated. On 2 February 1960 she had an episode of collapse with pallor, sweating, unrecordable pulse and blood pressure. She responded initially to hydrocortisone and mephentermine but despite continued steroid therapy she had two similar episodes of circulatory collapse with hypotension, and died on 7 February.

BACTERIOLOGY The organism isolated from the faeces and blood was found to have the following antigenic structure: 0 – 6, 7; H – c : 1, 5, which is that of *S. cholerae suis;* the biochemical reactions were those of the Kunzendorf strain. The organism was sensitive to streptomycin, chloramphenicol, tetracycline, polymyxin, and sulphonamides.

NECROPSY

The relevant findings were as follows:—The heart weighed 920 g. and was greatly increased in size mainly through enlargement of both auricles, especially the left. Both ventricles were moderately dilated with slight hypertrophy of the musculature on the right. The left auricle was solid in consistence; plaques of calcification were present in the anterior wall and the dilated cavity was occupied for the most part by a mass of laminated thrombus partially adherent to the wall, the central two-thirds of which consisted of pinkish-brown pultaceous material, the appearances suggesting abscess formation. The pulmonary venous return had channelled between the auricular wall and the thrombus to reach the mitral valve which was extremely fibrosed and calcified, the orifice being reduced to the characteristic 'button-hole' slit, 1·4 cm. in length (Fig. 2). The tricuspid valve was also fibrosed and appeared to be functionally incompetent; the right auricle was considerably dilated and the muscle hypertrophied with prominent trabeculation of the wall. Aortic and pulmonary valves were healthy; section of the coronary arteries showed no significant lesion and there was no evidence of fibrosis of the ventricular myocardium. A small amount of clear serous fluid was present in each pleural cavity and the lower lobe of each lung was oedematous.

The liver (1,310 g.) showed the nutmeg mottling of chronic venous congestion.

Other organs showed no significant lesions; various sites, including spleen, kidneys, cerebral, mesenteric and limb arteries, were examined for evidence of embolic phenomena but none was visible. There was no ulceration of the intestines and Peyer's patches were not notably hypertrophied,

Material for culture was taken from the abscess in the left auricle, blood in the right ventricle, the spleen, the gall bladder, and from the proximal, middle, and distal parts of the lumen of the small intestine. Only one positive result was obtained; this was given by the swab from the auricular abscess, the culture yielding a pure growth of the organism isolated during life on three occasions in the patient's blood cultures.

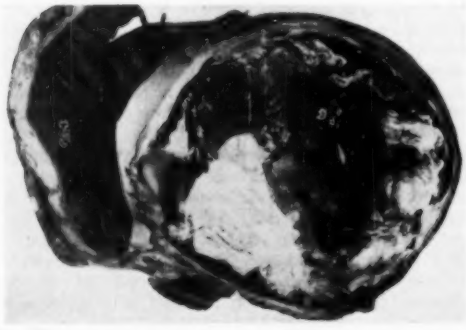

FIG. 2. *Heart from above. Transverse section through both auricles to show left auricular thrombus, the central part (site of abscess) being removed to show stenotic mitral valve. Note also dilatation and trabeculation of right auricle.*

HISTOLOGY

HEART Small masses of thrombus are present in the lumen of several of the lesser branches of the coronary circulation (Fig. 3). Probably in relation to these emboli there are microscopic areas of myocardial necrosis scattered diffusely and fairly frequently throughout the walls of both ventricles (Fig. 4). The extent of the necrotic lesion is such that it could have contributed to the general failure of the myocardium. Similar thrombotic emboli are present also in the spleen (Fig. 5). Sections of the left auricular wall confirm the presence of calcification. Also, the myocardial fibres are considerably diminished in number and replaced by dense fibrous tissue in which there are broad strands of collagen. The adjacent thrombus shows organization of varying age at the periphery while more centrally there is a mass of necrotic material containing many neutrophil polymorphs.

LIVER Sections show the pattern of venous congestion. Other organs sectioned show no significant histological abnormality. Specific staining of all sections shows no Gram-negative bacilli.

The cause of death was presumed to be congestive heart failure from a severe degree of mitral stenosis and incompetence, the failure being aggravated terminally by *S. cholerae suis* septicaemia with infection of a left auricular thrombus and multiple foci of myocardial necrosis of embolic origin.

DISCUSSION

This case shows features in common with that of Boycott and McNee (1936). In their case mitral

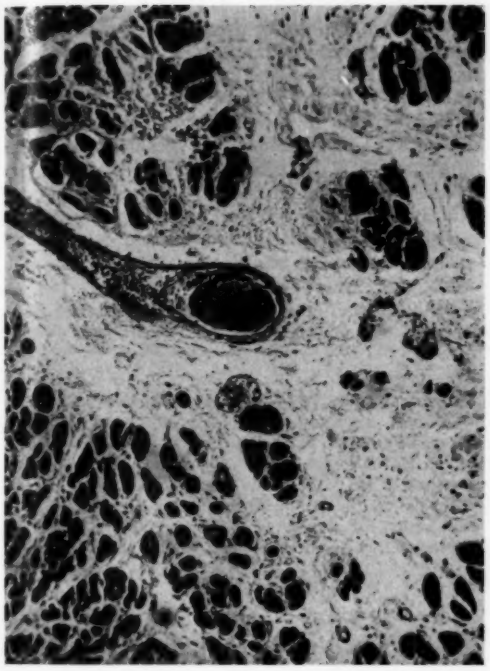

FIG. 3. *Section of myocardium. Embolus in small coronary vessel.* (*Haematoxylin and eosin* × *110.*)

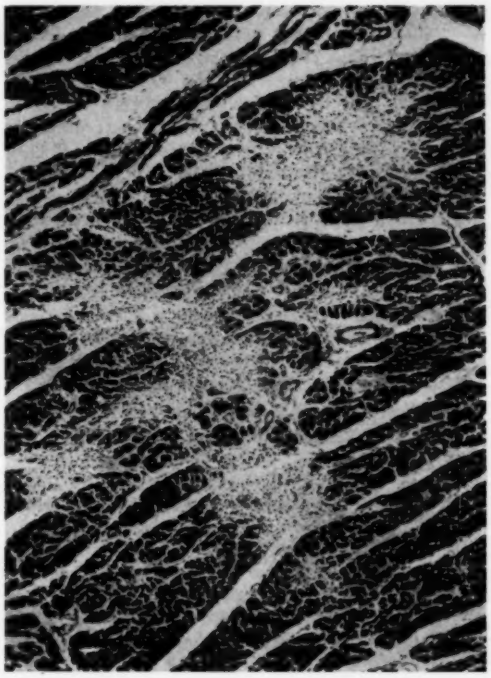

FIG. 4. *Section from wall of left ventricle. Areas of necrosis of myocardial fibres.* (*Phosphotungstic acid-haematoxylin.* × *50.*)

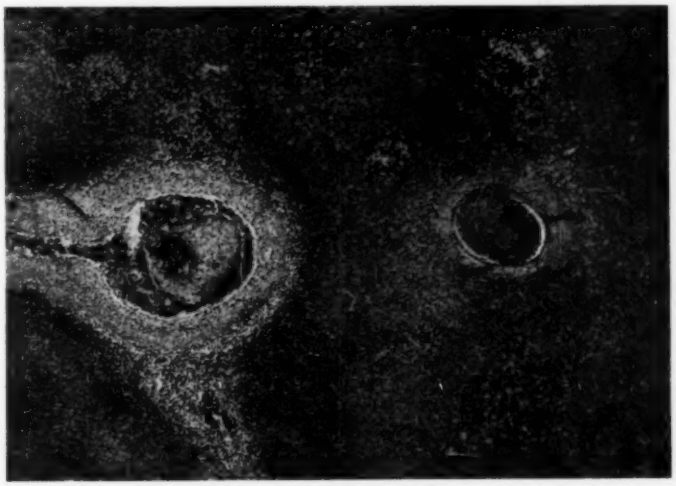

FIG. 5. *Section of spleen. Two small arteries plugged by emboli.* (*Haematoxylin and eosin* × *30.*)

stenosis was present and death followed an episode of circulatory collapse. Necropsy revealed laminated thrombus filling most of the left auricle and its appendage, the organism being isolated from the thrombus. This is the only instance in which *S. cholerae suis* infection in an auricular thrombus has previously been fully substantiated. Forster (1939), in describing a patient with *S. cholerae suis* bacterial endocarditis, commented on thrombus in the right auricular appendage but gave no details of its bacteriology. A case of infected ventricular thrombus was described by Jager and Lamb (1943), who found thrombus at the site of a previous myocardial infarction and were able to culture *S. cholerae suis* from it. Infected vegetations on valve cusps have been reported more frequently; Rich and St. Mary (1956) found 24 cases in the literature and reported a further case. In our case and in that of Boycott and McNee there was no evidence of vegetation on valve cusps.

The episodes of circulatory collapse and hypotension in our case and of circulatory collapse in Boycott and McNee's case resemble the shock-like state described in patients with Gram-negative bacteraemia (Aldridge, 1960) and in other septicaemic states (Altemeier and Cole, 1956). The latter authors described 93 cases exhibiting shock in association with serious infection, and among them mentioned one case of *S. cholerae suis* septicaemia. They suggest a number of possible mechanisms, including haemoconcentration, peripheral vasodilatation, and toxic effects upon the heart, adrenals, and medullary centres. Further, Reid (1957) postulates that a generalized Schwartzman phenomenon may be implicated in some cases. It seems probable that the shock is the consequence of liberation of bacterial toxins but the precise mechanism of its action is still speculative. Spink (1960) has suggested that usual doses of steroid may be inadequate in treating shock associated with infection and claims good results from using up to 1 g. of hydrocortisone daily for two or three days.

Mortality in *S. cholerae suis* infection is high, especially in adults. Saphra and Winter (1957) report a mortality of 20·3% in their series of 359 cases. It is unlikely that this mortality will be greatly reduced by antibiotics alone. Flippin and Eisenberg (1960) comment that for the most part treatment of the salmonelloses with antibiotics has been unsatisfactory. In *S. cholerae suis* infection there is a striking tendency for localized abscesses to form and antibiotics may fail to cure a proportion of cases for this reason. For instance, Fitzgerald, Snyder and Singleton (1959) described a patient with *S. cholerae suis* meningitis who relapsed six times after an initial clinical response to antibiotics and was cured after excision of an infected subarachnoid cyst.

The origin of the infection in the present case is not known; there were no known instances of infection in the family and no local cases had been reported by the Public Health Laboratory Service.

We wish to thank Dr. William Brockbank for permission to publish the case record, also Professor A. C. P. Campbell and Dr. R. W. Fairbrother for helpful advice and criticism. We are grateful to Dr. G. Storey for giving us access to his M.D. thesis on salmonellosis, to Dr. D. H. Payne, of the Public Health Laboratory Service, for establishing the identity of the organism, and to Mr. N. Mowat, F.I.M.L.T. for the photographs.

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Serum precipitin reactions in Hashimoto's thyroiditis

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SYNOPSIS Sera from certain patients with Hashimoto's disease or a related condition contain a non-precipitating antithyroglobulin which produces in double-diffusion tests with thyroglobulin a line of clearing in the agar. Two such sera are described.

Other sera from examples of the disease give negative precipitin tests with thyroglobulin but combine with sera containing 'clear line', non-precipitating antithyroglobulin to give a positive precipitin test. The serum from 15 out of 26 precipitin-negative cases of Hashimoto's disease showed this latent property. Eight of the 15 were tested by the tanned red cell technique and showed substantial titres of antithyroglobulin.

The presence of auto-antibodies in Hashimoto's disease was first described in 1956 (Roitt, Doniach, Campbell, and Vaughan-Hudson). Their importance as an aid to the diagnosis of the condition is generally accepted even if their role in producing cell damage is not. The test for precipitating antithyroglobulin is in common use but this antibody cannot be detected in many cases of the disease. The presence of non-precipitating antithyroglobulin in the serum of certain cases of Hashimoto's disease was described by Goudie, Anderson, and Gray (1959) who found that this antibody produced between serum and thyroglobulin 'wells' on an Ouchterlony plate a line of clearing in the agar's slight milky opacity. This was shown by chemical means to be a zone of high protein concentration. It was also noted by these workers that sera producing this phenomenon had enhancing properties, *i.e.*, when they were allowed to diffuse into the area of agar between a 'well' containing thyroglobulin and a 'well' containing serum from certain precipitin-negative cases of Hashimoto's disease, a precipitate appeared which did not do so in the absence of the enhancing serum.

This clearing phenomenon was observed independently in this laboratory and no doubt in others also. Two patients with such sera have been encountered, and one of these, patient L, has been observed over a period of three years and her thyroid gland examined histologically. The other, patient M, has been observed for a much shorter period. Serum from the latter gave a line of clearing unaccompanied by precipitate. That from patient L has shown a transition over the years through the phases (1) line of precipitate only, (2) line of precipitate plus line of clearing, (3) line of clearing only, and (4) no reaction. The sera from these patients will be named L and M. Their value as enhancing sera was compared and the effect of combining them was tested.

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METHODS

All precipitin tests were made on Ouchterlony plates at room temperature, using 0·75% Difco agar in physiological saline in which 0·03% sodium azide was incorporated to minimize contamination. The antigen used was a crude saline extract of thyroid gland obtained at operation. The gland was frozen, sliced thinly, and left at 4°C. for 48 hr. in three times its bulk of physiological saline. The supernatant fluid was centrifuged to remove particulate matter and was stored in the frozen state; it was diluted 1 in 4 in saline for use.

Two types of test were used. In the first (Fig. 1) the enhancing serum (in this case M) was set up alongside four precipitin-negative Hashimoto sera and allowed to diffuse into the area of agar between test sera and antigen. In the second, mixtures of enhancing and test sera in various proportions were set up directly against antigen.

The titre of 'clear line' non-precipitating antithyroglobulin in serum L and serum M was determined approximately by testing doubling dilutions of the serum against antigen, the titre being that dilution still giving a line of clearing.

Tanned red cell tests were made with Burroughs Wellcome reagents using the method recommended by the makers.

RESULTS

Figure 2 shows that an enhancing serum fails to affect the outcome when a strong precipitin is present in the

FIG. 1. *'Clear line' serum M used to detect by diffusion the latent precipitating component in precipitin-negative Hashimoto sera. Tc and Td are such sera giving a positive result: Te and Tf give a negative result. A = antigen.*

TABLE I

RESULTS OF PRECIPITIN TESTS IN PATIENTS WITH HASHIMOTO'S DISEASE

Group	*Number of Cases*	*Test for Precipitin*	*Test for Enhancement*	*Total in Group*
1	7	+++	–	20 (39%)
	5	+++	Not tested	
	2	+	–	
	3	+	+	
	1	+	Not tested	
	2	Clear band		
2	15	–	+	31 (61%)
	11	–	–	
	5	–	Not tested	

precipitin-negative cases out of a total of 26 tested were found to be enhanced. Sera L and M had no such effect on precipitin-negative sera from 88 patients without thyroid disease and from 33 with thyroid disease not of auto-immune type. Histological examination had shown the presence of Hashimoto's thyroiditis in several of the 15 patients whose sera were enhanced.

The very strong enhancing effect of serum L and serum M on one another is shown in Fig. 3. Their

FIG. 2. *'Clear line' serum M reacting alongside serum Ta and serum Tb, both of which contain strong precipitin. A = antigen.*

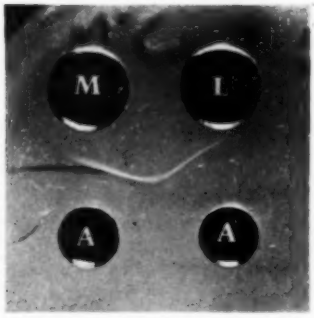

FIG. 3. *'Clear line' L and M sera set up alongside one another. A = antigen.*

test Hashimoto serum. The enhancing serum, however, does interact with certain precipitin-negative Hashimoto sera as already shown, and Table I summarizes the results of combining these by diffusion or direct mixture. The sera tested included all cases diagnosed as examples of Hashimoto's disease by physicians of this hospital's endocrine clinic. Many of these patients had been receiving treatment for the condition for a considerable time before the tests described were made.

Group 2 is the one of most interest. Sera from 15 'line of clearing' titres, determined by dilution as described above, were 1/16 (L) and 1/3 (M). Taking this difference into account, mixtures of L and M with Group 2 sera capable of enhancement did not give identical results when tested for precipitin, and this perhaps indicates a qualitative difference in the two (Table II).

Serum L and serum M both gave titres of 1/25,000 with the tanned red cell test, and eight of the sera from group 2 capable of enhancement which were tested gave titres ranging from 1/2,500 to 1/25,000.

TABLE II

COMPARISON OF ENHANCING EFFECTS OF SERA L AND M IN MIXTURES WITH PRECIPITIN-NEGATIVE HASHIMOTO SERA

Test Serum	*Serum M*	*Serum L*
1	−	+
2	+	+
3	−	+
4	−	+
5	+	+

CASE REPORTS

Patients L and M were a little unusual clinically as well as serologically.

M had splenomegaly and evidence of mild hypersplenism at the times serum was obtained. The reason for this was not elucidated.

L was considered to be a fairly typical case of Hashimoto's disease until the onset of rapid, painful, diffuse enlargement of the thyroid gland. Malignancy was suspected and substantial portions of each lobe were excised. The histological picture was reported by Dr. H. McD. Cameron to be that of struma reticulosa (Brewer and Orr, 1953). The serum component causing a line of clearing in agar was detected one year before the gland enlarged and persisted for six months after partial thyroidectomy.

DISCUSSION

The eight precipitin-negative sera tested for antithyroglobulin and capable of enhancement all showed substantial tanned red cell titres. Other sera with similar titres gave a positive precipitin reaction. Thus the negative precipitin test in certain cases of Hashimoto's disease is not explained by too low a serum level of antithyroglobulin.

It seems that sera from cases of Hashimoto's disease may react in gel precipitin tests positively to give a line of precipitate and/or a line of clearing in unclear agar, or negatively, to show no detectable change. Mixtures of sera reacting negatively also react negatively but when such sera are mixed with those capable of producing a line of clearing in agar the mixture in a majority of cases reacts positively in precipitin tests, a line of precipitation being obtained if optimum proportions of the constituents are chosen.

Such differences in antibody behaviour are usually ascribed to differences in avidity for antigen at different stages of an immune process. Slight chemical or physical differences in antibody or the presence or absence of complementary or inhibitory substances might explain 'differences in avidity'. From the less theoretical point of view it can be said that a precipitin-negative Hashimoto serum is serologically very similar to a precipitin-positive one, since in most cases it has a latent capacity to take part in a precipitin reaction with thyroglobulin.

I am grateful to Drs. T. J. Thomson, W. D. Smith, Campbell Love, and M. McNicol for the opportunity to examine sera from their patients and for their cooperation. I am indebted also to Dr. John S. Stevenson for encouragement and advice, to Mr. P. Waldie and his staff for photographs, and to Dr. H. McD. Cameron for permission to summarize his histological report.

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Minimal criteria for the diagnosis of megaloblastic anaemia of pregnancy

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SYNOPSIS The routine examination of buffy coat films is a valuable diagnostic aid to screening large numbers of specimens of blood and is applicable to hospital and general practice, provided that a bottle of sequestrinated venous blood can be examined in the laboratory within 24 hours.

The diagnosis of folic-acid deficiency anaemia of pregnancy can be made, in many cases, before the appearance of megaloblasts in the peripheral blood by finding macrocytes, polylobing of polymorphonuclear leucocytes, and 'out of step' haemoglobinization of normoblasts in the buffy coat film.

Accurate haemoglobin estimations at intervals throughout pregnancy, and particularly in the last three months, are an important and in some cases essential part of ante-natal care. Any haemoglobin found to be below 75% (11·1 g.%) should be followed by the examination of a series of buffy coat films while the anaemia is being treated.

It has been shown (Goodall, 1957) that an accurate diagnosis of megaloblastic anaemia of pregnancy can be made by examining films made from the buffy coat layer of centrifuged peripheral blood. Goodall's series was controlled by concurrent sternal marrow examination and he concluded that, although marrow examination was slightly more sensitive than the buffy film, the buffy film alone could supply the necessary information in most cases without subjecting the patient to the discomfort of a marrow puncture.

In this investigation an attempt has been made to discover the minimal information required for a diagnosis of megaloblastic anaemia of pregnancy, and so a record was kept of the haemoglobin, the reticulocyte count, the packed cell volume, and the appearance of the buffy coat film stained by the Leishman method.

The diagnosis of megaloblastic anaemia was not made until megaloblasts appeared in the peripheral blood but we now believe that the diagnosis can in fact be established before this time by taking into account the other haematological appearances and all relevant clinical findings.

Received for publication 10 December 1960.

MATERIALS AND METHODS

The examinations were carried out on venous blood taken from pregnant women at the ante-natal clinic or in the wards of the Princess Mary Maternity Hospital, Newcastle upon Tyne, over a period of 21 months, from November 1958 to July 1960 inclusive.

All the women in the series were delivered in the hospital although not all of them had received ante-natal care at the hospital clinic.

Specimens were collected in universal containers with Sequestrin (diamino-ethane-tetra-acetic-acid-dipotassium salt) as anticoagulant, and in most cases were not received in the laboratory until the following day, having been stored overnight either at room temperature or 4°C. and then transported by van.

When the blood specimen was received the haemoglobin was estimated by a cyan-methaemoglobin method using a modified Drabkin's solution (King and Wootton, 1956). If this was 75% (11·1 g.%) or below, or if the case was already being followed, the reticulocytes and packed cell volume were estimated and a buffy coat film was made.

The packed cell volume was estimated using a Hawksley micro-haematocrit centrifuge, after which the capillary tube containing the blood was cut and the buffy coat and some of the underlying red cells were used to make a smear which was stained with Leishman's stain and examined under a film of oil. We found that a buffy coat

film made in this way was a good representative sample of all the cells in the peripheral blood.

When examining the film several factors relating to the red cells and white cells were considered and recorded as being present or absent. No attempt was made at a quantitative estimation.

RED CELLS The red cells were examined for anisocytosis, polychromasia, macrocytosis, Howell-Jolly bodies, and nucleated forms. The nucleated forms were further divided into normoblasts, megaloblasts, and cells in which the degree of haemoglobinization was in advance of nuclear maturation. These are referred to as 'out of step' normoblasts. Only nucleated red cells with some degree of haemoglobinization were considered (Goodall, 1957).

WHITE CELLS White cells were examined for the presence of polylobing (hypersegmentation) of the nuclei of neutrophil polymorphonuclear leucocytes or giant forms of metamyelocytes and polymorphonuclear leucocytes. Hypersegmentation was taken to mean six or more clearly defined lobes in the nucleus.

RESULTS

In the 21 months covered by the survey there were 3,741 births at the maternity hospital, and of that number 45 of the patients were diagnosed as having megaloblastic anaemia during or immediately after their pregnancy. Of the 45 patients, 38 had a single delivery including one who delivered a hydatidiform mole. Five had twin deliveries and two had triplets. Most of the patients were multiparous but nine of them were experiencing their first pregnancy and eight were pregnant for only the second time.

The maternal ages ranged from 21 to 44 years.

It will be seen from Fig. 1 that the haemoglobin level at the time of diagnosis was most commonly between 55% and 60% (8·1 and 8·9 g.%). In only one case was the haemoglobin over 75% (11·1 g.%); this estimation was made after a blood transfusion and gives a wrong impression.

Fig. 2 indicates the time of diagnosis in relation to parturition and it is shown that all the cases except one were diagnosed in the last trimester of pregnancy or the first week of the puerperium. The diagnosis made at 19 weeks was that in the case of molar pregnancy referred to previously. Twenty-two of the cases were diagnosed on the first occasion that a buffy coat film was examined.

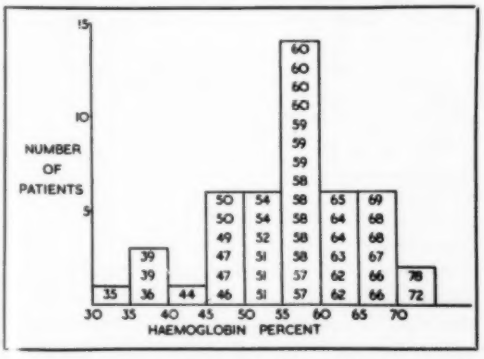

FIG. 1. *Histogram showing the haemoglobin level in the individual cases at the time of diagnosis. (Hb 100% = 14·8 g./100 ml.)*

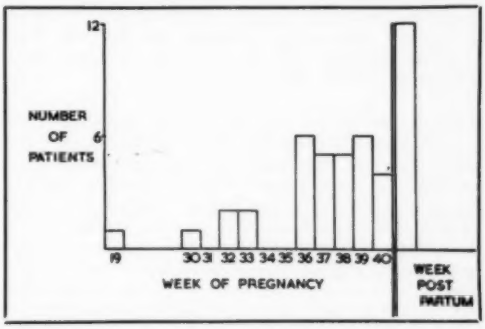

FIG. 2. *Diagram illustrating the relationship between megaloblastic anaemia of pregnancy and the week of pregnancy in which it was diagnosed.*

ILLUSTRATIVE CASES

CASE 32 (MRS. M. W.) This patient was 29 years old and in her fourth pregnancy. She was found to have megaloblasts in a buffy film at the thirty-third week of pregnancy on the first occasion that a film was examined (Fig. 3).

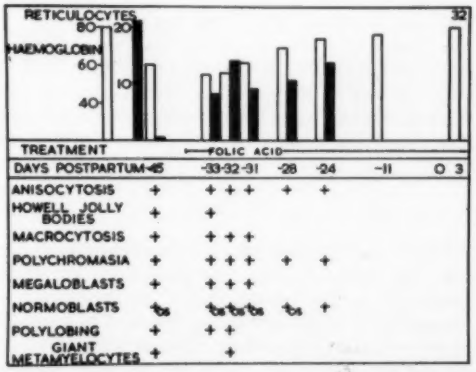

FIG. 3. *The time scale is in days post partum so that the minus sign indicates days ante partum. O.S. = Normoblasts with heamoglobinization in advance of nuclear maturation.*

She was treated with folic acid orally and by the time she reached her thirty-ninth week, the haemoglobin was 76% (11·3 g.%). Shortly after delivery it was 80% (11·8 g.%). No blood transfusion was required and there was no complication.

CASE 4 (MRS. E. A.) This patient, like the last, was 29 years old and in her fourth pregnancy. During the series it was found in several cases that the megaloblastic picture appeared after a course of intramuscular or intravenous iron injections. In this case (Fig. 4) a course of Imferon injections was followed by the appearance of megaloblasts in a specimen taken before delivery on the day of delivery. In spite of the Imferon the haemoglobin level had dropped slightly and the reticulocyte count remained at 1%. The anaemia was later treated with oral iron and folic acid.

CASE 39 (MRS. D. M.) This patient was 27 years old and was in her fourth pregnancy. On the first occasion that a buffy film was examined, more than six weeks before delivery, her blood picture was one which we came to recognize later as typical of folic-acid deficiency anaemia (Fig. 5). No megaloblasts were seen but the film showed macrocytes, polylobing of polymorphonuclear leucocytes, and 'out of step' haemoglobinization of normoblasts. During a course of Ferrivenin megaloblasts appeared, but only four days before the delivery and when the haemoglobin was 60% (8·9 g.%).

CASE 34 (MRS. C. W.) This patient is included as representative of the group of nine primiparae. She was 23 years old and was diagnosed in the first week post partum after having had a transfusion of 2 pints of blood at delivery. However, it is again seen that macrocytes, polylobing, and 'out of step' haemoglobinization were present in the peripheral blood a month earlier (Fig. 6) so that a diagnosis could have been made at the time.

There was one maternal death in our series of 45 patients. A woman of 21, who was pregnant for the first time, was admitted as an emergency with a fulminating pre-eclamptic toxaemia and died 12 days after delivery in renal failure, with a haemoglobin level of 35% (5·2 g.%). At necropsy it was considered that megaloblastic anaemia had played a part in the death.

DISCUSSION

While examining buffy films it became clear that folic-acid deficiency anaemia of pregnancy could be diagnosed, in many cases, before the appearance of megaloblasts in the peripheral blood. The presence of macrocytosis, polylobing of neutrophil polymorphonuclear leucocytes, and 'out of step' haemoglobinization of normoblasts was followed, in the absence of treatment with folic acid, by the full megaloblastic picture.

The advantage of using the other criteria when

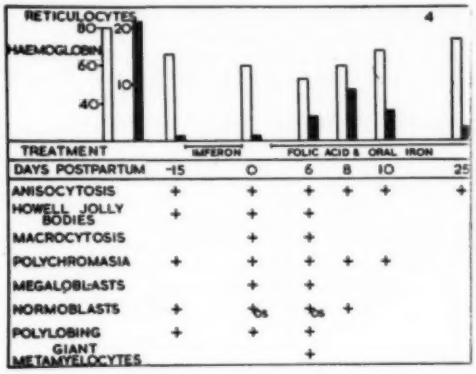

FIG. 4

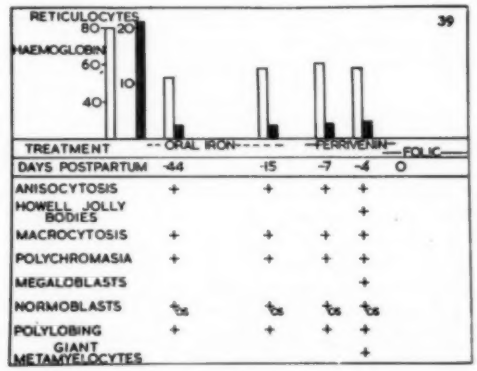

FIG. 5

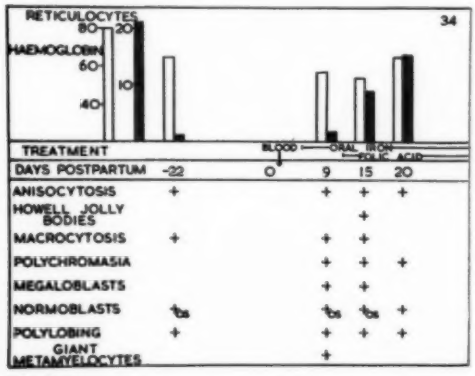

FIG. 6

FIGS. 4, 5, AND 6. *The time scale is in days post partum so that the minus sign indicates days ante partum. O.S. = Normoblasts with haemoglobinization in advance of nuclear maturation.*

present is that treatment can be started earlier than would otherwise be the case (Cases 34, 39).

The danger of masking a true Addisonian pernicious anaemia in pregnancy and consequently precipitating subacute combined degeneration of the cord by giving folic acid would be largely overcome by further investigations of any suspected case and medical supervision during folic-acid therapy. While not altogether condemning the use of folic acid as a routine prophylactic in pregnancy we feel that it is an unnecessary and costly refinement. Even if it were used in this way the need for a simple screening method would still exist because of such factors as poor absorption of folic acid from the bowel or neglect by the patient, resulting in too small a dose or perhaps none at all.

We should like to thank the consultant staff of the Princess Mary Maternity Hospital, Newcastle upon Tyne, for access to case notes.

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The July 1961 Issue

THE JULY 1961 ISSUE CONTAINS THE FOLLOWING PAPERS

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Conventional voltage electrophoresis for formiminoglutamic acid determination in folic acid deficiency J. KOHN, D. L. MOLLIN, and L. M. ROSENBACH

The urinary excretion of assayable vitamin B_{12} and radioactivity after parenteral ^{58}Co B_{12} in man J. F. ADAMS

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Studies on the blood lipids and lipoproteins in thalassaemia and sickle cell anaemia C. CHOREMIS, V. KYRIAKIDÈS, and E. PAPADAKIS

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Plasma magnesium in health and disease SAMIR HANNA

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J. clin. Path. (1961), **14**, 540

Antihaemophilic factor deficiency, capillary defect of von Willebrand type, and idiopathic thrombocytopenia occurring in one family

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SYNOPSIS Thrombocytopenia of the type found in idiopathic thrombocytopenic purpura is described in a family with a deficiency of antihaemophilic factor, and a capillary abnormality. The propositus (father) has thrombocytopenia and abnormal platelet morphology, together with a plasma deficiency of antihaemophilic factor, but normal capillaries. His two daughters each are deficient in antihaemophilic factor with normal platelets but abnormal capillaries.

In 1931, von Willebrand described a familial haemorrhagic disorder which he called hereditary pseudo-haemophilia. The bleeding diathesis, inherited as an autosomal Mendelian dominant, was associated with excessive haemorrhage following minor injury and with spontaneous bleeding from the skin and mucous surfaces. The prolonged bleeding time and increased capillary fragility were accompanied by a normal platelet count and morphology, and a normal blood coagulation time and clot retraction. Dogmatic views of its pathogenesis were not given at that time.

In recent years this disease has been more intensively studied. A number of workers have described cases associated with deficiency in antihaemophilic factor (Larrieu and Soulier, 1953; Alexander and Goldstein, 1953; Quick and Hussey, 1953; Soulier and Larrieu, 1954; Ingram, 1956; Schulman, Smith, Erlandson, Fort, and Lee, 1956; Nilsson, Blombäck, Jorpes, Blombäck, and Johansson, 1957; Nilsson, Blombäck, and Francken, 1958; Biggs and Macfarlane, 1958; Horler and Witts, 1958; Wilkinson, Nour-Eldin, and Israëls, 1958; Blackburn, 1961), while two of Blackburn's (1961) series had a deficiency of Christmas factor in addition to the basic capillary abnormality of von Willebrand's disease. Braunsteiner (1955) and Braunsteiner and Pakesch (1956) showed that cases of von Willebrand's disease and those with abnormal platelet function, as demonstrated by electron microscopical studies and thromboelastography, may occur in the same family. So far as we are aware, however, thrombocytopenia of idiopathic type has not been described before in families of this group. The two daughters in the present family have been included in Blackburn's (1961) series of 158 cases of primary capillary haemorrhage.

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LABORATORY METHODS

The three affected members of the present family (father and two daughters) were investigated using the following methods.

For the bleeding time, an Ivy method, as described by Biggs and Macfarlane (1957a), was used and the tourniquet test by the method of Bell, Lazarus, and Munro (1940).

Microscopy of nail-fold capillaries was carried out as described by Macfarlane (1941). The capillaries were examined for increased tortuosity, variation in calibre, and defective contractility to trauma. The platelet count was done by the method of Lempert (1935), and the whole blood clotting time by that of Lee and White (1913). The one-stage prothrombin test was one slightly modified from that described by Biggs and Macfarlane (1957a). Prothrombin consumption was estimated by the method of Merskey (1950). The thromboplastin generation test was carried out by the method of Biggs and Douglas

(1953), and antihaemophilic factor titration by the method of Pitney (1956).

Standard methods were used for the determination of the haemoglobin level, red cell and total white cell counts and the differential white cell count.

THE PATIENTS

FATHER He is an auditor, aged 34 years, who, when referred to hospital, gave no history of taking cytotoxic drugs or of exposure to any toxic industrial hazard. There was a clear history of excessive bruising for about 14 years, and he had always bled abnormally after receiving cuts or abrasions. There had never been spontaneous haemorrhage from mucosal surfaces, but transient crops of small blue spots had been noticed under the armpits. Nettlerash had been observed from time to time on the shoulders. He had never had joint symptoms. At the age of 5 years he required blood transfusion after the removal of his tonsils and adenoids. He had been told that he bled for many days after this operation and that the bleeding stopped for one and a half days at one stage during this episode. After dental extractions he has always bled profusely for 12 hours and has continued to bleed for up to seven days in spite of local dental attention. One year previously he had been transfused with two bottles of plasma after dental extractions. His past illnesses included chickenpox, measles, mumps, tonsillitis, sandfly fever, and malaria.

He and his two daughters are the only members of the family who have been affected by abnormal bleeding. He has three brothers (one of whom has two sons), and one childless sister. His father, three paternal uncles and three paternal aunts and their children, his mother, one maternal uncle with no children and two maternal aunts, one of whom has two daughters and one son, are also unaffected by abnormal bleeding.

Laboratory findings The haemoglobin, red and white cell counts (including differential), red cell morphology, one-stage 'prothrombin' time, whole blood clotting time, bleeding time, tourniquet test, and capillary microscopy of the nail folds were all within the normal range. The platelet count was low (70,000 per c.mm.) and hypogranularity and many giant and small forms were seen. The prothrombin consumption index was high, 100% (normal well below 40%). The thromboplastin generation test showed a plasma deficiency, and the titre of antihaemophilic factor was low, 30% (normal range 50 to 150%). On previous occasions the platelet count had been as low as 20,000 per c.mm. and the bleeding time prolonged to 18 minutes. Bone marrow examination showed an increased number of abnormal megakaryocytes with maturation arrest.

DAUGHTER AGED EIGHT YEARS She has always bled excessively following cuts and abrasions, and has bruised with abnormal ease. Recurrent and troublesome epistaxes began at the age of 5 years. Small crops of purpura on the trunk and limbs have been noted from time to time. Dental extractions have always led to abnormal haemorrhage. On one occasion the removal of one tooth necessitated admission to hospital for one week, but transfusion was not necessary. The extraction of two teeth on another occasion resulted in admission to hospital for three weeks, and the need for blood transfusion. She has never had haemarthrosis.

Laboratory findings Haemoglobin, red cells, white cells, and platelets were normal. The bleeding time was 10 minutes (normal less than five and a half minutes), the tourniquet test negative, and the whole blood clotting time seven to nine minutes (normal). The thromboplastin generation test showed a deficiency in a plasma factor. The one-stage prothrombin time was normal. The prothrombin consumption index was 39% (probably abnormal). The antihaemophilic factor titre was low, namely 30%, 35%. Capillary microscopy of nail folds showed that tortuosity and calibre variation was considerably increased with defective contractility to trauma of some forms.

DAUGHTER AGED FOUR YEARS She has always bruised abnormally easily and has bled excessively following cuts and abrasions. A plasma transfusion was necessary on one occasion when three teeth were extracted. Purpura and haemarthroses had not been noticed by her parents, but the former was present on examination in small crops over the shoulders as well as widespread bruising of the trunk and limbs.

Laboratory findings The haemoglobin, red cells, white cells, and platelets were normal. The bleeding time was nine and a half minutes (prolonged), the tourniquet test was positive, the whole blood clotting time 10 minutes (normal), the one-stage prothrombin time was normal, the prothrombin consumption index abnormal (50%), and there was a deficiency in the patient's plasma fraction in the thromboplastin generation test. The antihaemophilic factor titre was low (25%). Capillary microscopy of nail folds showed increased tortuosity and variation in calibre with defective contractility to trauma of some forms.

DISCUSSION

While a mild to moderate degree of thrombocytopenia is well known to occur in some haemorrhagic phases of von Willebrand's and allied diseases, it does not persist in between these episodes as it has done in the father of the two girls.

The precise significance of the vascular abnormality, thrombocytopenia, and plasma deficiency in antihaemophilic factor in this family is obscure at present. It would seem that most cases of von Willebrand's disease are inherited as an autosomal dominant (Biggs and Macfarlane, 1958; Pitney and Arnold, 1960; Blackburn, 1961) but this is not always the case (Blackburn, 1961). While the inheritance of the antihaemophilic factor deficiency in the father and two daughters in the present family follows such a pattern, the platelet and vascular abnormalities clearly do not do so. These three main

abnormalities may show variable penetrance and expressivity as manifestations of a single affected gene. Alternatively they may be due to the expression of different genes which may be located on the same chromosome.

Recently Raccuglia and Neel (1960) described kindred of 311 individuals in whom Dutch ancestry predominated. The variable manifestations of haemorrhagic disorder included a prolonged bleeding time, a morphological defect in platelets, and antihaemophilic factor deficiency. Genetic analysis suggested that the bleeding tendency was determined by a single dominant gene of variable penetrance and expressivity. A 22-year-old Caucasian man was shown by Perry, Opfell, and Baker (1960) to have plasma deficiencies of antihaemophilic factor and of plasma thromboplastin antecedent factor in association with increased vascular fragility. Different members of his family, however, had isolated abnormalities of each of the three types mentioned and also variable combinations of all three defects.

We are indebted to Dr. Barbara Lawson for carrying out preliminary tests on the girls.

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J. clin. Path. (1961), **14**, 543

Coagulation tests on capillary blood

A screening procedure for use in small children

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SYNOPSIS A method of investigating the coagulation mechanism is described.

Results obtained by this procedure are compared with those of tests carried out on venous blood obtained simultaneously from normal subjects and patients with a variety of clotting defects. Good correlation was found between the results of P and P tests on venous and capillary blood, and also between thromboplastin screening tests on capillary blood and antihaemophilic and Christmas factor levels.

The methods described have proved reliable as a pre-operative screening procedure in routine use over a period of nearly two years.

Investigation of the coagulation mechanism is most reliably carried out on blood obtained rapidly from a clean venepuncture. In small infants and some older subjects, however, such specimens may be difficult to obtain; in such cases, free-flowing capillary blood is preferable to a venous sample obtained with difficulty through a small needle, in which contamination with tissue fluid and delay in taking are likely to result in early changes of coagulation before citrate is added. In this paper we describe a procedure by which a routine investigation of the blood coagulation mechanism can be carried out on 0·2 ml. of capillary blood; the results are compared with those obtained on venous blood taken from the same subjects simultaneously.

PRINCIPLES

Blood samples used for coagulation studies should be mixed with anticoagulant without delay in order to arrest the coagulation process as soon as possible. The most effective way of doing this in the case of capillary blood is to take a small measured quantity of blood directly into several times its own volume of diluting fluid containing sodium citrate; by this means, sufficient blood to provide nearly 2 ml. of diluted plasma can be obtained and the coagulation process halted within a matter of seconds. Such samples can be tested by any procedures requiring the use of dilute plasma. For the initial examination of samples from patients suspected of having a coagulation disorder, we have chosen to use a modification of the thromboplastin screening test of Hicks and Pitney (1957) as a test of blood thromboplastin formation, and the prothrombin and proconvertin (P and P) test of Owren and Aas (1951) as a test of the extrinsic coagulation system. This combination of tests will detect deficiencies of all known clotting factors except fibrinogen, as shown in Table I. As a simple test to exclude serious fibrinogen deficiency, the clotting time test of Dale and Laidlaw (1911) may be carried out on the capillary blood sample.

TABLE I

RESULTS OF P AND P AND THROMBOPLASTIN SCREENING TESTS IN VARIOUS HAEMORRHAGIC STATES

Deficiency	*P and P*	*Thromboplastin Generation Screening Test*
Hageman factor Plasma thromboplastin antecedent Christmas factor (factor IX) Antihaemophilic factor (factor VIII) Factor V	Normal	Abnormal
Factor VII Prothrombin	Abnormal	Normal
Stuart-Prower factor (factor X) Combined deficiency of liver disease, vitamin K deficiency, etc.	Abnormal	Abnormal
Fibrinogen	Normal	Normal

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If the screening procedures mentioned above reveal a defect, more specific tests may be carried out on the same sample; in this way, the exact nature of the clotting defect may be identified and its degree determined by means of quantitative assay procedures. This paper, however, is concerned with screening procedures only, and the details of specific assay methods will not be considered.

METHODS

SUBJECTS STUDIED Capillary blood and venous blood were collected as described below from normal adults and from patients of various ages with haemophilia, Christmas disease, or congenital deficiency of factor V, factor VII, or plasma thromboplastin antecedent (PTA). Blood was also taken from a series of adults on long-term anticoagulant therapy with phenindione (Dindevan) and from patients with acquired 'hypoprothrombinaemia' from various causes, particularly liver disease.

COLLECTION OF CAPILLARY BLOOD Anticoagulant-diluting fluid, 1·8 ml., of the following composition is measured into a capped polythene tube (60 × 15 mm.):

Trisodium citrate	2 g.
Sodium chloride	5·6 g.
Barbitone buffer, *p*H 7·35 (Owren, 1949)	200 ml.
Distilled water	800 ml.

Of this fluid 0·2 ml. is then drawn up from the polythene tube into a pipette calibrated to contain 0·2 and 0·4 ml. A well-warmed finger (or heel in the case of an infant) is stabbed with a sharp Hagedorn needle, and, after wiping away the first drop, blood is drawn up under the fluid in the pipette until the total volume is 0·4 ml. The whole is then washed out into the remainder of the fluid in the polythene tube, which thus contains 2·0 ml. of a 1 in 10 dilution of whole blood in buffered citrate-saline. Two heparinized microhaematocrit tubes are then filled from the same stab wound. The whole collection should not take more than one minute.

The dilute blood is centrifuged at 2,500 r.p.m. for 15 minutes, and the supernatant used in the tests described below. The subject's packed-cell volume (P.C.V.) is determined by the use of a microhaematocrit centrifuge, and used to calculate the actual dilution of the plasma tested (see below).

COLLECTION OF VENOUS BLOOD Nine parts of blood, obtained by clean venepuncture within a few minutes of taking the capillary sample, are mixed with one part of 3·1% trisodium citrate and centrifuged at 2,500 r.p.m. for 15 minutes.

PROTHROMBIN AND PROCONVERTIN (P AND P) TEST This was carried out by the method of Owren and Aas (1951) as modified by Biggs and Macfarlane (1957). Capillary plasma was tested after removal of the red cells by centrifugation as described above, but without further dilution. Citrated venous plasma was tested at a 1 in 10 dilution in the following solution:—

Trisodium citrate	1·5 g.
Sodium chloride	5·6 g.
Barbitone buffer, *p*H 7·3 (Owren, 1949)	200 ml.
Distilled water	800 ml.

The same final concentrations of citrate, NaCl, and buffer were thus obtained in the test dilutions of both venous and capillary plasma.

The clotting times in this system of the test plasmas were determined in duplicate in each case, and the mean clotting times converted to percentages of normal 'P and P' activity by interpolation on a standard dilution curve prepared from pooled normal venous plasma. In order that the results obtained on capillary and venous blood might be directly compared, the percentage activity of a 1 in 10 plasma dilution was calculated in each case by correcting the observed activity for the diluting effect of the anticoagulant, which varies with the P.C.V.; the calculations involved may be reduced to the following formulae:

$$\text{I}\quad \text{Capillary P and P (\%)} = \frac{\text{Pc} \times 100}{(100 - \text{P.C.V.})}$$

$$\text{II}\quad \text{Venous P and P (\%)} = \frac{\text{Pv}\ (100 - 0{\cdot}9\ \text{P.C.V.})}{90 - 0{\cdot}9\ \text{P.C.V.}}$$

where Pc and Pv are the P and P percentage activities respectively of the capillary and venous plasma dilutions tested. In practice, it is always necessary to correct capillary plasma results in this way, as differences in P.C.V. have a significant effect on the final plasma dilution. Similar differences have little effect on the final dilution of venous plasma, as the acutal 1 in 10 dilution is carried out in this case on citrated plasma and not on whole blood; the correction factor is only applied here in order that the venous and capillary results may be directly comparable, and is unnecessary for the interpretation of venous results as such.

THROMBOPLASTIN SCREENING TEST In most instances, the method of Hicks and Pitney (1957) was used, with Inosithin[1] (0·02 g. per 100 ml.) as the source of phospholipid; capillary plasma was tested after removal of the red cells without further dilution. During the period of this study, it was found that greater reproducibility of the results of this test could be achieved by adding an optimum amount of kaolin to the mixture of dilute plasma and Inosithin, and incubating at 37°C. for five minutes before the addition of calcium chloride. Some of the subjects seen towards the end of the study were tested by this modification of the standard procedure.

As the results of this test are not capable of expression in an accurate quantitative manner, no attempt was made to compare results obtained on capillary blood directly with those obtained on venous samples from the same subject, as in the case of the P and P test. Capillary blood from at least one normal control subject was tested in parallel with each sample obtained from a patient, and the second-stage clotting times compared directly.

ANTIHAEMOPHILIC FACTOR ASSAYS Four of the 16 haemophilic patients were infants from whom venous blood could not easily be obtained; in these cases, antihaemophilic factor assays were carried out on capillary blood.

[1]Associated Concentrates, Woodside, L.I., N.Y.

In all the other cases of haemophilia investigated, antihaemophilic factor assays were performed on venous samples by a modification of the thromboplastin generation test similar to that described by Pitney (1956), in which the ability of the patient's $Al(OH)_3$-adsorbed plasma to correct the defect of $Al(OH)_3$-adsorbed plasma from a severe haemophiliac was compared with that of $Al(OH)_3$-adsorbed pooled normal plasma.

CHRISTMAS FACTOR ASSAYS In each of the seven patients with Christmas disease studied, the diagnosis was based on the result of a thromboplastin generation test, and the level of Christmas factor was determined by a one-stage method in which the correction of the kaolin clotting time of Christmas-factor-deficient plasma by serial dilutions of patient's plasma was measured.

RESULTS

EFFECT OF DELAY IN COLLECTING CAPILLARY BLOOD It was thought that a likely source of error in results obtained in capillary blood would be delay in the collection of blood after pricking the finger; this might allow some of the early changes of coagulation to occur at the site of injury before the sample was obtained. In order to determine the importance of speed of collection, three separate capillary blood samples were taken consecutively from the same fingerprick in a small series of subjects, and thromboplastin screening tests and 'P and P' determinations were carried out in parallel on all three specimens. The time taken to collect each specimen varied between 20 and 60 seconds, and the whole collection was completed in each case within three minutes.

It was found that there was no significant difference in 'P and P' results between any of the three specimens nor between the first two specimens in the results of the thromboplastin screening test; in this test, however, the clotting times obtained with the third specimen were often significantly longer than those with the first two. Typical sets of results are shown in Table II. These findings indicate the importance of rapid collection of capillary blood samples, and suggest that specimens collected within one minute will give reliable results in practice.

TABLE II

RESULTS OF TESTS ON THREE CONSECUTIVE CAPILLARY BLOOD SAMPLES FROM SAME FINGERPRICK

Subject	*Blood Sample*	*Thromboplastin Screening Test* — *Incubation Time (min.)* — *Clotting Time (sec.)*				*P and P (%)*
		2	4	6	8	
A	I	22·5	12	15·5	20·5	89
	II	80	12	13·5	17	85
	III	105	29	15	19	85
B	I	71	13·5	15	16	82
	II	48	14	15	—	78
	III	41	15	15·5	—	82

PROTHROMBIN AND PROCONVERTIN (P AND P) TEST The P and P test was performed on samples of venous and capillary plasma obtained simultaneously from 21 normal subjects, 40 patients receiving anticoagulant treatment with phenindione, and 10 patients with various congenital clotting defects. The results, corrected for dilution, are plotted in Fig. 1, from which it can be seen that there is good correlation between the capillary and venous results, particularly in the 0 to 60% range.

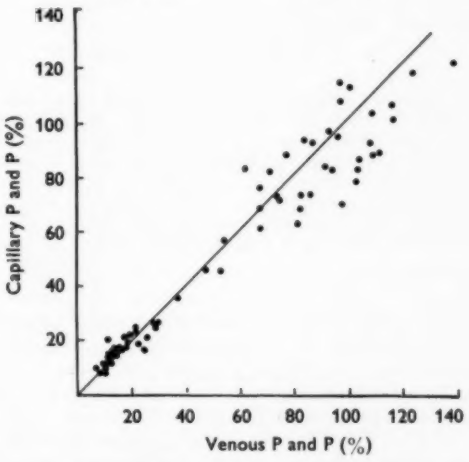

FIG. 1. *Correlation between 'P and P' results obtained on venous and capillary blood samples. The diagonal line represents 100% correlation. The coefficient of correlation between the two sets of readings is 0·969.*

THROMBOPLASTIN SCREENING TEST Thromboplastin screening tests were carried out on a total of 29 capillary blood samples from patients with haemophilia and Christmas disease of various degrees of clinical severity, in whom antihaemophilic factor or Christmas factor assays were also performed. In eight instances, the antihaemophilic factor or Christmas factor had been raised to values between 15 and 50% by replacement therapy. In Fig. 2, the shortest second-stage clotting times obtained in the thromboplastin screening test are plotted against the antihaemophilic factor and Christmas factor levels;

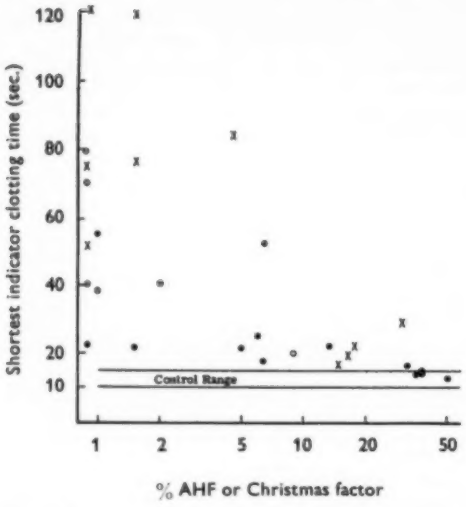

FIG. 2. *The relationship between the minimal second-stage clotting time in the thromboplastin screening test performed on capillary plasma and the level of antihaemophilic or Christmas factor.* ●, *haemophilia (antihaemophilic factor assay on venous plasma);* ○, *haemophilia (antihaemophilic factor assay on capillary plasma);* ×, *Christmas disease. All the normal controls gave minimal clotting times between 10 and 14 seconds in the thromboplastin screening test.*

the screening test times were outside the normal control range in every case in which the antihaemophilic factor or Christmas factor was below 35%, and tended to be longest in the cases with the lowest levels of antihaemophilic factor or Christmas factor.

In Figs. 3 to 5 are shown the results of 'P and P' and thromboplastin screening tests carried out on capillary blood samples from patients with 'hypoprothrombinaemia' resulting from vitamin K deficiency and with congenital deficiencies of factor V and factor VII respectively. These examples illustrate the three patterns of abnormality detectable by the use of these two tests (cf. Table I).

Many children have been investigated by these methods before operation, on account of a history suggestive of a bleeding tendency. Whenever the shortest clotting time in the thromboplastin screening test exceeded that of the normal control by three seconds or more, or the 'P and P' level was below 50%, further investigation of the coagulation mechanism was carried out. All other patients were regarded as suitable for operation provided that the platelet count and bleeding time were also normal, and none of these bled excessively.

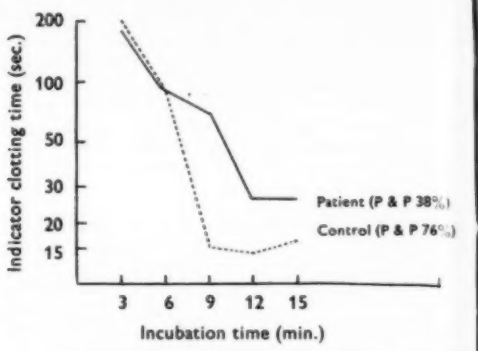

FIG. 3. *Thromboplastin screening test on capillary plasma from a patient with vitamin K deficiency.*

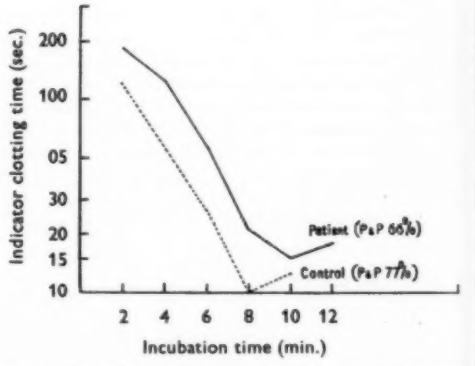

FIG. 4. *Thromboplastin screening test on capillary plasma from a patient with congenital factor V deficiency.*

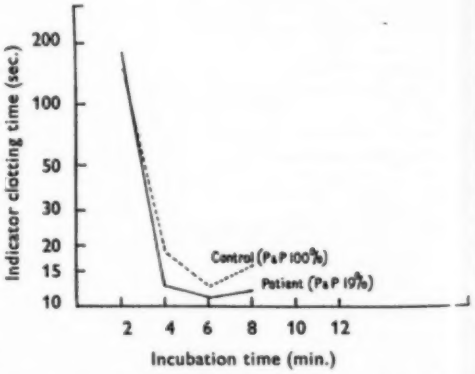

FIG. 5. *Thromboplastin screening test on capillary plasma from a patient with congenital factor VII deficiency.*

DISCUSSION

The main theoretical objections to the use of capillary blood for tests of the coagulation mechanism concern the difficulty of obtaining a large enough volume of blood before the early changes of coagulation have occurred; this difficulty is aggravated by the likelihood of contamination of the sample with tissue fluid. The method described here, in which a very small volume of blood is taken directly into anticoagulant, largely overcomes this obstacle by ensuring rapid collection; the reliability of the method is shown by the fact that two consecutive samples could always be obtained in this way without significant change in clotting activity.

Somewhat similar methods of collection have been used by other workers to obtain blood for prothrombin time estimations (Dyggve and Lund 1954; Miale and Winningham, 1960), but those who have attempted more extensive studies of the coagulation process on capillary blood have usually taken larger volumes. Ashley (1958), for example, took 0·9 ml. of capillary blood into 0·1 ml. citrate from a small series of normal subjects and obtained results in a battery of coagulation tests which were comparable with those of similar tests on venous blood from the same subjects; he showed no direct evidence, however, of the reliability of his procedure as a means of detecting coagulation defects.

The methods described here have proved their value in practice, as they have been used with complete success over a period of nearly two years as a pre-operative screening procedure in children suspected of having a bleeding tendency. During this period, seven cases of haemophilia, two of Christmas disease, and one of PTA deficiency have first been detected by these methods. That abnormal results were obtained in all cases in which the level of antihaemophilic or Christmas factor was known to be below 35% indicates that degrees of deficiency of these factors likely to be associated with excessive bleeding at operation would not be missed by the use of these tests on capillary blood.

One of the chief objects of screening tests of coagulation is to anticipate the need for special measures when surgery is indicated, and it must be emphasized that the results of such tests, whether they be carried out on capillary or venous blood, should always be interpreted in the light of a careful clinical history. Whenever abnormal results are obtained, or clinical considerations suggest that near-normal results are not to be relied upon, additional, more specific tests should be carried out in order to reach an exact diagnosis. Semiquantitative tests for individual clotting factors may be performed on the same capillary sample by determining its ability to correct plasmas with known specific deficiencies in appropriate test systems, and in most instances the diagnosis can be made in this way. This is not to suggest, however, that results obtained on capillary blood are as reliable as those on blood obtained from a clean venepuncture; if they are readily obtainable, venous samples should always be used for detailed coagulation studies, especially in the assessment of mild disorders.

We should like to thank Dr. J. L. Stafford for allowing us to investigate patients in the anticoagulant clinic at St. George's Hospital, London, and haematologists at various other hospitals who have permitted us to investigate patients with bleeding disorders. We are indebted to Mrs. Janet Macpherson, A.I.M.L.T., for much valuable technical assistance, and to Dr. J. A. Fraser Roberts for help with statistics.

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The anaemia of lead poisoning

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SYNOPSIS Three cases of lead poisoning with a hypochromic anaemia, despite adequate iron stores, are presented and discussed.

The anaemia of lead poisoning is usually thought to be normochromic and normocytic, and punctate basophilia is regarded as a constant feature of the well-developed case. Amongst six proven cases of chronic lead poisoning seen in this Department in the last few years, two of the patients were not anaemic and in a third the degree of anaemia was very slight. We propose to comment here only on the remaining three patients, all of whom showed well-established anaemia. In all three the peripheral blood findings were those of iron deficiency; in only one case was punctate basophilia found.

Formerly the anaemia in lead poisoning was attributed to a shortened survival of cells in the circulation but more recently the importance of interference with haemoglobin synthesis has been recognized, and this is demonstrated in two of our three patients in whom an inability to use available iron despite active eyrthropoiesis was an important factor in the development of the anaemia. Only in the third did the haemolytic element predominate.

CASE HISTORIES

C.S., a lead burner, aged 41 years, who had twice previously been treated for plumbism, complained of increasing weakness of his hands. After investigation lead poisoning was again diagnosed. The blood findings initially were Hb, 8·7 g./100 ml. (59%), P.C.V., 30·5%, M.C.H.C., 28·5%, R.B.C.s, 4·2 m./c.mm., M.C.V., 73 cμ, M.C.H., 21 μμg., reticulocytes, 7·5%. Punctate basophilia was not seen. The serum iron level was 137 μg./100 ml. and the total iron-binding capacity 260 μg./100 ml. Marrow examination showed normoblastic erythropoiesis with excess stainable iron. The uptake of ^{51}Cr T½ was 26 days; on electrophoresis of the serum there was no apparent reduction of the haptoglobin. Urinary urobilinogen levels were 3·4, 6·5, and 7·7 mg./24 hours on three different days. Faecal urobilinogen measured on two occasions was 265 mg. and 687 mg./24 hours. The M.C.H.C. remained between 28·5% and 29·5% until oral treatment with versene began. The patient then had a lead diuresis and the blood values gradually rose to Hb, 13·5 g./100 ml. (91%), P.C.V., 46·5%, and M.C.H.C., 31·25%.

R.C., a lead burner, aged 45 years, presented with abdominal pain and a gross blue line on the gums. Blood findings were Hb, 9·3 g./100 ml. (63%), P.C.V., 32·75%, M.C.H.C., 28·5%, R.B.C.s, 4·3 m./c.mm., M.C.V., 76 cμ., M.C.H., 21·75 μμg., and reticulocytes, 5·5%. Punctate basophilia was not seen. The serum iron was 78 μg./100 ml. and the total iron-binding capacity 450 μg./100 ml. Urinary urobilinogen levels were 5·7 and 11·9 mg./24 hours on two occasions. Urinary coproporphyrins showed a daily excretion of over 1,000 μg. Stainable iron was present in the marrow in spite of the peripheral hypochromia and punctate basophilia was seen in red cell precursors. The uptake of ^{51}Cr T½ was 32 days and the plasma haptoglobin 145 mg./100 ml. (normal 45-150 mg./100 ml. as methaemoglobin).

M.S., a housewife, aged 30 years, had taken large quantities of lead and opium pills for diarrhoea for many years. The blood lead level was 80 μg./100 ml.; there was a blue line on the gums and the daily urinary coproporphyrin excretion exceeded 700 μg. Blood findings were Hb, 7·8 g./100 ml. (53%), P.C.V., 26·25%, M.C.H.C., 30%, and reticulocytes, 22%. Punctate basophiles were readily found only in buffy coat smears. The serum iron level was 160 μg./100 ml. and the total iron-binding capacity 335 μg./100 ml. The urinary urobilinogen level was 9·3 mg./ 24 hr., and the uptake of ^{51}Cr T½, 18 days. Marrow examination showed normoblastic erythropoiesis with a very great excess of stainable iron. The patient responded satisfactorily to versene which greatly increased the excretion of lead in the urine. The blood values rose to Hb 12·4 g./100 ml. (84%), P.C.V., 40%, and M.C.H.C., 31%.

DISCUSSION

More than one mechanism has been blamed for the production of the anaemia of lead poisoning. The red cells may be rendered more 'brittle' by the lead (Aub, Fairhall, Minot, and Reznikoff, 1925) and therefore may have a shorter life span than normal

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Lead poisoning may interfere with attempts to compensate for this haemolytic state by inhibiting either the incorporation of iron into the protoporphyrin nucleus or the production of the porphyrin part of haem (Eriksen, 1955; Goldberg, Ashenbrucker, Cartwright and Wintrobe, 1956). The utilization of iron would therefore be prevented either directly or indirectly. In all our three cases it seems probable that increased red cell destruction was concerned in the genesis of the anaemia as shown by the raised reticulocyte count and, in the third case, a very active haemolytic process was the chief cause. In the other two cases, although neither the ^{51}Cr $T\frac{1}{2}$ nor the haptoglobin concentration was reduced, the increased urobilinogen excretions confirmed the reticulocytosis in suggesting that some degree of increased blood destruction was present. More significant, however, was the finding of a peripheral hypochromia in all three cases despite adequate stores of iron in the bone marrow. This indicated that haemoglobin synthesis was selectively depressed and by thus lagging behind red cell formation materially contributed to the production of anaemia in each of these three cases of lead poisoning.

We are indebted to Dr. E. B. Hendry and Dr. A. Goldberg for pigment excretion results and to Professor E. J. Wayne and Dr. J. A. W. McCluskie for other details of the cases.

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The recognition of Hageman deficiency in blood donors

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SYNOPSIS The laboratory findings on a blood donor whose blood coagulation time was greatly prolonged are described. The defect was caused by a deficiency of Hageman (contact) factor, the nature of the defect being demonstrated by a simple glass adsorption test using normal serum.

Abnormally prolonged coagulation of human blood *in vitro* due to a deficiency of Hageman factor was first described by Ratnoff and Colopy (1955). It is a rare familial trait, known to affect both sexes, but is not associated with a haemorrhagic tendency *in vivo*. Margolis (1957), Biggs, Sharp, Margolis, Hardisty, Stewart, and Davidson (1958), and Soulier, Wartelle, and Ménaché (1958) have contributed to our understanding of the part played by Hageman factor in the activation of the coagulation mechanism. It is believed by the above workers that Hageman factor is normally present in the inactive phase in plasma, but is itself activated by contact with glass after which it reacts with plasma thromboplastin antecedent, thus initiating further stages of thromboplastin generation.

The importance of recognizing Hageman factor deficiency lies in its differentiation from other coagulation disorders during the performance of tests *in vitro*.

In a deficiency which produces no clinical abnormality, a true picture of its incidence is more likely to emerge from an investigation of the normal population. The National Blood Transfusion Service regularly collects blood samples from many thousands of normal volunteers and occasional cases of Hageman deficiency may be encountered. Although the abnormality would pass unnoticed in the bottle containing anticoagulant, it is the practice to take a clotted blood sample from each donor for laboratory tests and it is on this sample that the defect may be recognized. It is the purpose of this report to describe such a finding and to detail a simple test by which the nature of the defect may be demonstrated.

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CASE REPORT

A blood donor, Miss S., aged 19 years, had given two donations without any abnormal findings. On the third occasion it was found that 24 hours after the donation only a trace of fibrin clot was present in the laboratory sample. An attempt to remove 'serum' with a pasteur pipette was followed five minutes later by the formation of a firm clot throughout the whole specimen. A recalcified plasma clotting time, made with the plasma from the main donation, was grossly prolonged and Miss S. was recalled a few days later for further investigation. The donor stated that she had suffered severe haemorrhage following tonsillectomy six years ago and that she tended to ooze blood for a few days after extraction of a tooth. At no time, however, had any haemorrhage necessitated a blood transfusion. She appeared fit and well. There was no family history of bleeding tendencies.

LABORATORY RESULTS

Bleeding time (Duke's method)	4 min. 30 sec.
Coagulation time (Lee and White 4-in. tube test at 37°C.)	a trace of fibrin appeared in each tube at about 17 min.
One-stage prothrombin time	16 sec. (control 14·5 sec.)
Prothrombin consumption index	100%

THROMBOPLASTIN GENERATION TEST Substitution tests using the donor's serum or plasma separately showed slightly abnormal results. When the donor's alumina plasma and serum together were incubated with platelets and calcium, thromboplastin generation was grossly abnormal.

PLATELETS Numerous platelets that appeared morphologically normal were present in the stained film.

FURTHER TESTS The history and laboratory investigations suggested the possibility of Hageman deficiency. Margolis (1958) found that Hageman factor may be adsorbed onto glass from which it could not be removed by subsequent washing with saline.

A clean tube was rinsed with a few drops of normal serum and then thoroughly washed six times in isotonic saline. The excess saline was shaken out and the tube placed in a water bath at 37°C. Miss S. plasma (collected in the proportion of nine parts of blood to one part of 3·8% sodium citrate), 0·2 ml., was added and re-calcified by adding 0·1 ml. of M/40 calcium chloride solution. The clotting time of this plasma was 1 min. 40 sec. with a firm clot appearing. A control test in an untreated tube showed only a trace of fibrin 20 min. after re-calcification. It is apparent that the defect in Miss S. blood lies in the absence of Hageman factor.

Investigation showed that the serum of Miss S. was unable to sensitize glass and correct her own plasma clotting time. Her parents had normal plasma clotting times and both could sensitize glass and correct their daughter's clotting deficiency. The defect could also be corrected by contact with glass activated by normal rabbit serum but duck serum was inactive.

STABILITY OF HAGEMAN FACTOR Investigation with the plasma of Miss S., using the principle of adsorption of Hageman factor onto the interior of clean glass tubes followed by washing in saline, showed that a high concentration of Hageman factor could be demonstrated in dried plasma (prepared from time expired donor blood), dried serum, and fresh deep frozen plasma (used in the treatment of haemophilia). No activity could be detected with human fibrinogen prepared by the method of Kekwick and Mackay.

Hageman factor could not be adsorbed onto silicone-coated glass, was not removed by absorption with aluminium hydroxide for three minutes, and was not inactivated by heating normal serum for 20 minutes at 56°C. Repeated washing of a sensitized tube with saline failed to remove the factor, but it was easily removed by washing with N/10 sodium hydroxide solution.

A titration of Hageman factor in a normal serum, using silicone-coated apparatus to prepare the dilutions, showed that a dilution of 1 in 500 of normal serum in saline would sensitize glass and reduce the subsequent plasma clotting time of Miss S. from 20 minutes to 3 minutes 20 seconds with the formation of a good clot. It is apparent that a very high concentration must be present in the original serum.

Two preparations of brain thromboplastin, one human and one rabbit, prepared by the acetone extraction method of Quick would both strongly sensitize glass and clearly contained potent contact factor. No activity, however, could be demonstrated in human saliva.

In a further experiment a single glass tube was sensitized by rinsing with a 1 in 100 dilution of stored normal serum followed by washing six times with isotonic saline. Miss S. plasma, 0·2 ml., was added and the tube incubated at 37°C. for four minutes. The plasma was then poured into a second unsensitized clean tube and re-calcified. The initial sensitized tube was washed twice in saline and a second sample of Miss S. plasma added and incubated for four minutes followed again by transference to a second clean tube and re-calcification. This process was repeated four times. The results are shown in Table I. It is clear that the action of Hageman factor is catalytic, and that activation will take place in deficient plasma, even though the source of factor is removed before adding calcium.

TABLE I

CORRECTION OF PLASMA CLOTTING TIME OF HAGEMAN-DEFICIENT PLASMA USING TWO STAGES

		Calcium Clotting Time
1	Controls: Hageman-deficient plasma in untreated tube	More than 20 min. (poor clot)
2	Hageman-deficient plasma in untreated tube after pre-activation for 4 minutes in sensitized tube	3 min. 35 sec. (good clot)
	As 2 above. Second application of Hageman-deficient plasma to same sensitized tube	2 min. 43 sec. (good clot)
	As 2 above. Third application of Hageman-deficient plasma to same sensitized tube	3 min. 10 sec. (good clot)
	As 2 above. Fourth application of Hageman-deficient plasma to same sensitized tube	3 min. 2 sec. (good clot)

DISCUSSION

Unless the possibility of Hageman deficiency is kept in mind, individuals who are affected by this abnormality may not be detected. Miss S. donated blood on two occasions without detection in the laboratory and it is probable that on both occasions the laboratory samples of her blood came into contact at some time with a glass surface that was 'clean' but nevertheless sensitized with contact factor from the blood of a previous donor. The experimental evidence shows how potent the contact factor is in normal serum and it is apparent that only the briefest exposure to a sensitized surface is necessary to correct the defect.

The normal serological laboratory procedure of using saline-washed pasteur pipettes for the separation of serum and cells will lead to immediate activation of the clotting mechanism, as indeed must have happened on the first and second occasions that Miss S. donated. It is vital that all tubes, pipettes, etc., used in the investigation of possible cases of Hageman deficiency should be cleaned in strong alkali before use.

The greatly prolonged plasma clotting time followed by correction when the test is repeated in a saline washed tube 'sensitized' with normal human serum enables the nature of the defect to be demonstrated very simply.

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J. clin. Path. (1961), **14**, 552

Renal enlargement in chronic cor pulmonale

P. A. ELLIS

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SYNOPSIS Examination of the post-mortem records of 1,391 unselected cases showed that the commonest condition associated with excessive diffuse enlargement of the kidneys was chronic cor pulmonale. A less striking enlargement was found in some cases of chronic rheumatic heart disease.

A hypothesis is put forward in which the renal enlargement is regarded as a work hypertrophy brought about by the kidneys' efforts to compensate for a chronic respiratory acidosis.

Very little is to be found in the literature on the subject of structural kidney changes in chronic cor pulmonale. Black and Stanbury (1958) comment on this lack of information and describe two patients with cor pulmonale in both of whom the last illness was associated with renal failure. Simpson (1957) also described renal failure as occurring, along with liver damage, in patients suffering from severe anoxia. Anatomical changes were present in one of Black and Stanbury's cases and were interpreted as being the earliest changes of tubular necrosis. Their other case also showed some enlargement of the kidney as a whole (combined weight 420 g.) and a small tuberculous focus was present. Simpson states that no anatomical lesions were present to account for the renal failure associated with anoxia.

The present investigations were undertaken as the result of an impression gained while performing routine post-mortem examinations that very large kidneys are often found in cases of chronic cor pulmonale.

MATERIAL

The records of 1,391 necropsies were examined. This total was made up of all the necropsies performed at Newcastle General Hospital in 1956 and 1957 (1,197) and 194 personal cases examined in 1958 and 1959.

Cases were admitted to the series when chronic lung disease was present along with enlargement of the right ventricle. All those with left ventricular enlargement or valvular disease were excluded.

RESULTS

Thirty-seven cases satisfied these criteria and Fig. 1 shows the distribution of their combined kidney weights. It will be seen that there were eight excee-

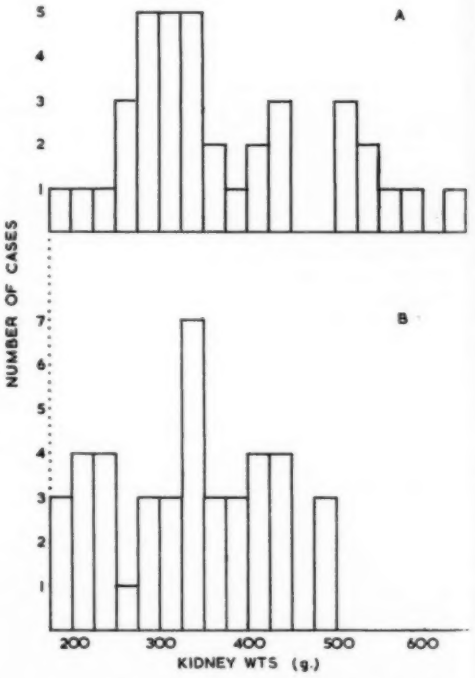

FIG. 1. *A Histogram showing distribution of the combined weights of the kidneys in 37 cases of cor pulmonale; B In 42 cases with right ventricular hypertrophy from causes other than pulmonary disease (all rheumatic except for one case of A.S.D.). Interval 25 grams.*

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ing 500 g., the modal value being about 310 g. In the rest of the 1,391 necropsies the kidney weighed more than 500 g. in 28 cases and in 22 of these the enlargement was due to purely local renal disease, including polycystic disease, pyogenic infections, solitary cysts, hypernephroma, secondary carcinoma, leukaemic infiltration, tubular necrosis, and amyloidosis. In the remaining six no acceptable local lesion was present. Of these, death was due to cardiac infarction in four, to cerebral tumour in one, and to head injury in one.

It is evident from these figures that enlargment of the kidneys to a combined weight of more than 500 g. occurs more often in chronic cor pulmonale than in any other condition, when purely local renal disease is excluded.

CORRELATION OF HEART AND KIDNEY WEIGHTS In Fig. 2 kidney weights are plotted against heart weights. Any increase in heart weights in these cases is, because of the criteria for inclusion in the series, the result of right ventricular hypertrophy. The correlation which is shown between heart and kidney weights, therefore, suggests a correlation between right ventricular weight and kidney weight, and consequently between kidney weight and the degree and duration of pulmonary disease.

The first mechanism that suggests itself as responsible for the renal enlargement is back pressure due to chronic congestive cardiac failure. If this were the only mechanism, one would expect to find a similar degree of renal enlargement in cases of chronic cardiac failure from other causes. Mitral stenosis was chosen for comparison because this condition commonly produces pure right ventricular hypertrophy associated with repeated episodes of congestive cardiac failure as does cor pulmonale.

In Fig. 3, 16 cases of pure mitral stenosis, all with enlargement of the right but with normal left ventricles, are similarly plotted. While there is some correlation between heart and kidney weight in Fig. 3 it appears that cases of mitral stenosis with hearts of a given weight have smaller kidneys than cases of cor pulmonale; and though the kidneys of one case reach 500 g. none exceed it. The numbers, of course, are smaller.

A large number of cases of rheumatic heart disease were excluded from the graph because the left ventricle was enlarged, due as a rule to aortic valve disease or to mitral incompetence. Figure 1b shows the distribution of kidney weights in all cases of rheumatic heart disease in which the right ventricle was enlarged, irrespective of other lesions. A single case of congenital heart disease with right ventricular enlargement was also included. Comparison of the two histograms clearly shows that there is a greater tendency to diffuse renal enlargement in chronic cor pulmonale.

A significant but less extreme degree of renal enlargement sometimes occurs in rheumatic heart disease (three cases with kidneys weighing between 475 and 500 g.).

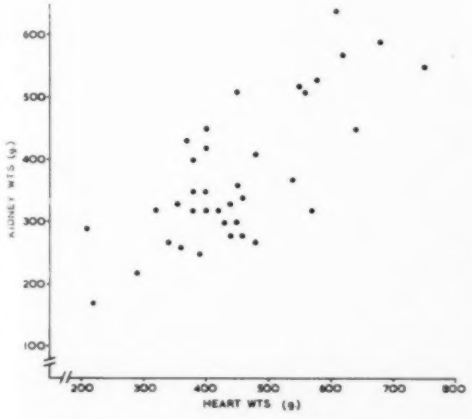

FIG. 2. *Scatter diagram showing kidney weights plotted against heart weights in 37 cases of cor pulmonale all having enlarged right, but normal left, ventricles.*

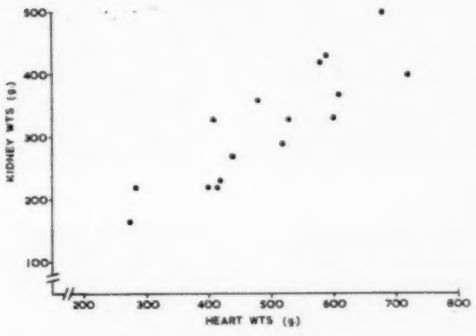

FIG. 3. *Scatter diagram showing kidney weights plotted against heart weights in 16 cases of pure mitral stenosis all having enlarged right, but normal left ventricles.*

MORBID ANATOMY OF THE ENLARGED KIDNEYS To the naked eye, except for their size, the intact kidneys appeared normal. They were firm to the touch in common with kidneys of chronic congestive failure

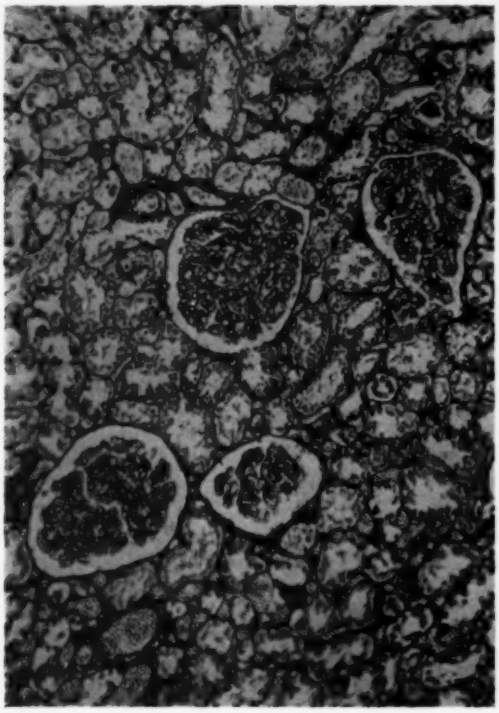

FIG. 4

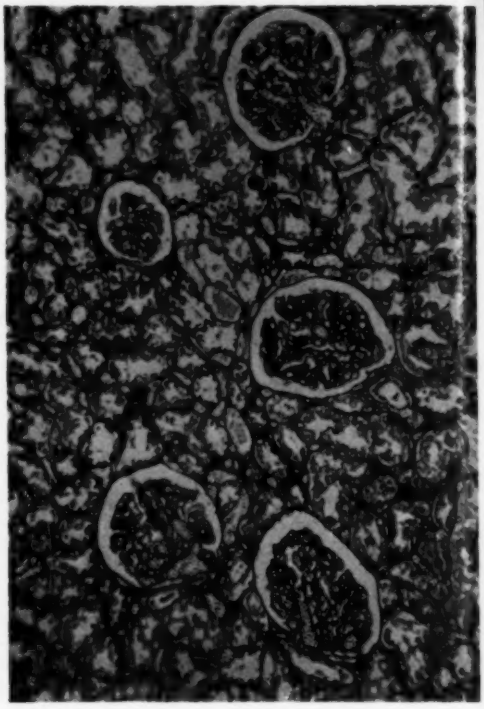

FIG. 5

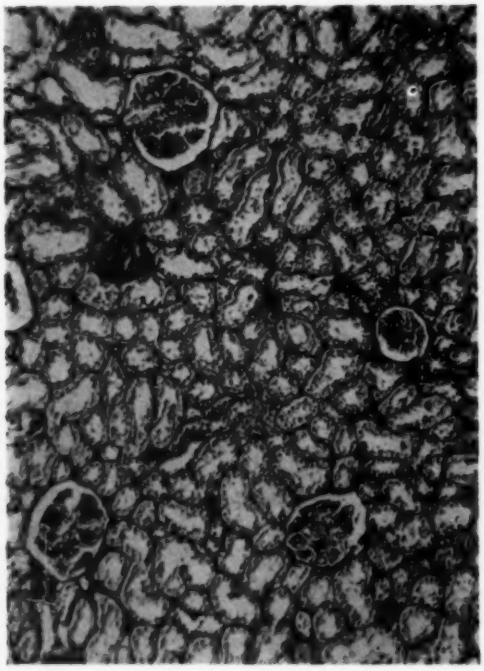

FIG. 6

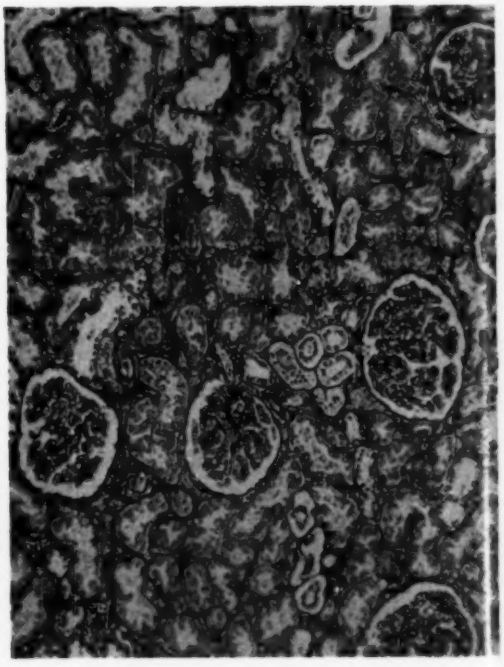

FIG. 7

FIGS. 4, 5, 6, AND 7. *Figs. 4 and 5 show cortex from two cases of cor pulmonale, Fig. 6 is a normal control, and Fig. 7 shows the cortex of a kidney hypertrophied (weight 320 g.) because of removal of its fellow many years before the patient's death. All* × 75.

in general. On cross section the cortex appeared broad (up to 1 cm. in thickness) and there was, as a rule, some degree of congestion.

Histological material was available from six cases in which the kidneys had weighed more than 500 g. and examination showed a striking and consistent increase in the diameter of the glomeruli (Figs. 4 to 7). In all six the diameters of 50 glomeruli were measured and the mean diameter and standard deviation calculated. Similar measurements were also taken from six normal kidneys and from the remaining kidney from a patient who died many years after a nephrectomy (see Table I). Detailed

TABLE I

DIAMETERS OF 50 GLOMERULI

Name	*Mean (μ)*	*Standard Deviation (μ)*	*Diagnosis*
WAD	255	32	Cor pulmonale
AIR	244	30	Cor pulmonale
REY	224	33	Cor pulmonale
HOD	218	24	Cor pulmonale
MGU	232	30	Cor pulmonale
BAR	263	38	Cor pulmonale
MEL	181	29	Normal
DOD	184	23	Normal
CUT	167	24	Normal
BEL	172	24	Normal
FAI	182	24	Normal
ESM	158	19	Normal
MAC	205	28	After nephrectomy

histological assessment of the tubules was not possible because of post-mortem autolysis which was advanced in some cases and present to some extent in all.

In none of the six cases was there a significant degree of nephron loss, though in all of them occasional glomeruli were hyalinized or fibrosed. Measurement of tubular diameters was not attempted as it appeared that a very large number of measurements would have to be taken to make the figure representative of the whole kidney and it was felt that the increase in tubular diameter could best be appreciated by means of photomicrographs (Figs. 4, 5, 6, and 7).

INCIDENTAL FINDINGS In the present 37 cases of cor pulmonale active chronic peptic ulcers occurred in no fewer than 10 and a healed chronic ulcer in one. Three patients had given a past history of gastrointestinal haemorrhage. The ulcers were multiple in three of the ten.

DISCUSSION

It might be argued that this increase in renal weight is entirely the result of congestion from chronic right ventricular failure. If this were so, the degree of renal enlargement should be approximately the same in cases with equal degrees of right ventricular enlargement, whether due to mitral stenosis or to primary lung disease. Figs. 2 and 3 were constructed in an attempt to refute this argument.

Kidney weights were available in only two patients dying of constrictive pericarditis, a condition in which passive venous congestion is prolonged and intense. The first, a man aged 51 years, had kidneys weighing 290 g., and in the second, a woman aged 62 years, the kidneys weighed 300 g. These figures argue against chronic congestion as the operative factor in the renal enlargement of cor pulmonale.

The following hypothesis is put forward as a possible mechanism. It is well known that when the total bulk of renal tissue is reduced, for instance by nephrectomy or by widespread nephron loss in chronic nephritis, the remaining nephrons become hypertrophied. That such hypertrophy is advantageous to the body's economy is obvious but the mechanism whereby it is brought about is obscure. Presumably it results from the accumulation of some substance or substances excreted or detoxicated by the kidney.

In chronic pulmonary disease associated with carbon dioxide retention the kidney is required to excrete an increased amount of acid; this it does by means of two mechanisms:—

1 An increase in hydrogen ion output results in an increase in the titratable acidity of the urine. The hydrogen ion is produced in the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3$, the first part of which is accelerated by the carbonic anhydrase of the renal tubular cells (Pitts and Alexander 1945; Pitts, Lotspeich, Schiess, and Ayer, 1948). One might expect this reaction to be pushed to the right by an increase in carbon dioxide tension in accord with the law of mass action. 2 An increase in ammonia excretion, ammonia being formed in the tubule cells from glutamine and certain amino-acids by the action of specific enzymes. By accepting a hydrogen ion each molecule of ammonia becomes an ammonium ion (NH_4^+). Once formed, the ammonium and hydrogen ions within the tubular cells change places with filtered sodium in the tubular lumen (Van Slyke Philips, Hamilton, Archibald, Futcher, and Hiller, 1943). The net result is a loss of hydrogen ion, either free, or in combination with ammonia as ammonium ion; an increase in the concentration of plasma bicarbonate ion HCO_3 (formed in the above reaction); and sodium reabsorption to balance the increase in bicarbonate.

An increase in the plasma bicarbonate raises the *p*H according to the Henderson-Hasselbalch equation

$$pH = 6{\cdot}1 + \text{Log}\left[\frac{BHCO_3}{H_2CO_3}\right]$$

Stated in another way, an increase in concentration of the bicarbonate ion causes a decrease in hydrogen ion concentration by pushing the reaction $H^+ + HCO_3 \rightleftharpoons H_2CO_3$ to the right, *i.e.*, it retards the ionization of carbonic acid (law of mass action).

It is known that in subjects made acidotic by the administration of NH_4Cl by mouth, NH^+_4 output in the urine rises rapidly in the first few hours as the urine becomes more acidic (Ferguson, 1951), after which it rises more gradually over several days. Davies and Yudkin (1952) rendered rats acidotic for periods of up to 30 weeks by giving N/20 HCl as drinking water. By means of studies on kidney slices they were able to demonstrate an increase in renal glutaminase, glycine oxidase and L. amino-acid oxidase, enzymes responsible for the formation of ammonia from glutamine, glycine, and L. leucine respectively. The gradual rise in ammonia output in chronic acidosis is thought to be due to this enzyme increase.

These observations indicate that the kidney tubule is able to respond to the stimulus of acidosis by increasing its content of those enzymes whereby the acidosis may be reduced, and, while in none of the experiments quoted above was renal hypertrophy mentioned, it is perhaps not entirely unjustifiable to suggest that it might occur, provided that the stimulus of an increased acid load were sufficiently prolonged.

The mechanism whereby glomerular enlargement is brought about cannot so easily be explained. Presumably it results in an enhanced rate of glomerular filtration in the presence of a falling cardiac output. In this connexion Davies and Kilpatrick (1951) have shown that in cor pulmonale with congestive failure, and in congestive failure from other causes, the effective renal plasma flow is reduced. The glomerular filtration rate is also decreased but not to the same extent, the filtration fraction (glomerular filtration rate/effective renal plasma flow) being raised.

It is possible that the above hypothesis may explain not only the renal enlargement of cor pulmonale but also the less striking enlargement seen in some of the cases of rheumatic heart disease. Gaseous exchange in the rigid lung of severe, long-standing mitral stenosis is known to be inefficient with regard to oxygen: however, the handling of the more diffusible carbon dioxide is said to be unaffected (Wood, 1956).

Several writers have commented on the association between chronic chest disease and peptic ulceration, the latter being present in about 20% of cases (Green and Dundee, 1952; Latts, Cummins, and Zieve, 1956). Flint and Warrack (1958) have shown that acute peptic ulceration can be found in a higher proportion of necropsies on cases of cor pulmonale if the lesions are looked for carefully. This is in agreement with the findings in the three cases described above.

The incidence of multiple chronic ulcer is remarkably high (four out of 10) but in so small a series this might be due to chance. The frequency of ulceration is probably related to a high acid output by the stomach. The hydrogen ions secreted by the parietal cells are believed to be produced in the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3$, the first part of which is catalysed by gastric carbonic anhydrase. As postulated above, for the same reaction occurring in the kidneys a high carbon dioxide tension would push this reaction to the right and increase acid production (Latts *et al.*, 1956). Though the exact relationship between hypersecretion of gastric hydrochloric acid and peptic ulceration is not yet clear, it is widely recognized that an association exists.

My thanks are due to Miss Georgina Hunt for preparing the sections and to Mr. E. Manns for the photomicrographs. I also wish to thank Dr. B. E. Tomlinson for advice and criticism while the paper was in preparation.

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Technical methods

A contact slide method for the isolation of staphylococci

I. A. PORTER AND JOYCE NICHOLSON *From the Department of Bacteriology, The Medical School, King's College, Newcastle upon Tyne*

The gauze-reinforced agar slab method introduced by Foster (1960) to assess environmental contamination was subjected to trial in the laboratory. In principle this method was sound but in our experience the gauze-reinforced agar slabs were difficult to prepare and far from easy to manipulate. A modification of Foster's method involving the use of agar-layered slides has been developed and employed successfully for more than six months in an investigation in which information was required about staphylococcal contamination of floors and other surfaces in a surgical ward (McNeill, Porter, and Green).

MATERIALS AND METHOD

MEDIUM Mannitol salt agar was employed and colonies of staphylococci could be recognized on this selective medium by their yellowish pigmentation after incubation at 37°C. for 36 to 48 hours.

PREPARATION OF CONTACT SLIDES Petri dishes each containing a glass slide 3 in. × 1½ in. are sterilized in the hot air oven (160°C.) for one hour. Strips of 1 in. Sellotape, 4½ in. long, are cut and one is placed, adhesive side upwards, over the bottom of each petri dish, the slide being removed with sterile forceps to permit this. The slide is then replaced lengthwise along the strip of Sellotape to which it adheres. The portions of Sellotape which project from each end of the slide are doubled on themselves to form handles by means of which the slide can be removed from the petri dish when required. Then 4·5 to 5·0 ml. of the melted agar medium is run onto the surface of the slide by means of a pipette. This amount of medium is found to cover the surface of the slide without spilling over its edges. It is essential to use petri dishes with flat bottoms.

USE OF CONTACT SLIDES A slide is removed from its petri dish by means of the Sellotape handles and turned through 180° so that the agar can be applied to the floor or other surface to be tested. Slight pressure is exerted on the slide to ensure that the agar makes contact along its entire length with the test surface. The slide, agar side uppermost, is returned to its petri dish and incubated in a moist atmosphere at 37°C. for 36 to 48 hours. Colonies of staphylococci are picked off into nutrient broth, incubated at 37°C. for 20 hours, and tested for coagulase production by the tube method. Those staphylococci found to be coagulase positive are then subjected to antibiotic and phage typing.

By the use of mannitol salt agar slides environmental contamination of wards and ancillary rooms by staphylococci has been demonstrated. It has been found possible by this means to isolate staphylococci from blankets and bedding, floors and windows sills, and from bed tables and lockers. The advantages of mannitol salt agar contact slides lie in the fact that they are easily and quickly prepared and economical in the amount of medium required; they can be handled without difficulty and they are efficient for the isolation of staphylococci.

No doubt similar slides layered with other suitable selective media could be used in investigations involving the isolation of other organisms causing environmental contamination.

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A micro-method for measuring glucose using the Autoanalyzer and glucose-oxidase

CYNTHIA WINCEY AND VINCENT MARKS *From the Department of Chemical Pathology, King's College Hospital Medical School, London*

Current methods for the estimation of glucose concentration in body fluids using the Autoanalyzer employ non-specific colour reactions. We have therefore devised a procedure using glucose-oxidase which permits of the determination of true glucose concentrations with this instrument. Glycolysis is prevented by the addition of formalin. The reagents for each estimation cost approximately 1½d.

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REAGENTS

1 SODIUM ACETATE BUFFER 0·5M Add 40% sodium hydroxide to about 800 ml. distilled water containing approximately 30 ml. glacial acetic acid until the *p*H is 5·0. Add water to bring the volume to 1 litre and recheck the *p*H.

2 O-TOLIDINE One per cent in absolute alcohol.

3 PEROXIDASE[1] Use 20 mg. per 100 ml. in acetate buffer. Reagents 2 and 3 are stable for several months in the refrigerator.

4 BUFFERED ENZYME-DYE REAGENT This is made up of reagents 1, 2, and 3. To about 80 ml. buffer add 5 ml. peroxidase solution, 1·0 ml. o-tolidine solution, and 1·0 ml. Fermcozyme.[2] Make up to 100 ml. with buffer. This reagent is stable for up to one month when refrigerated. It enters the instrument through tube 5.

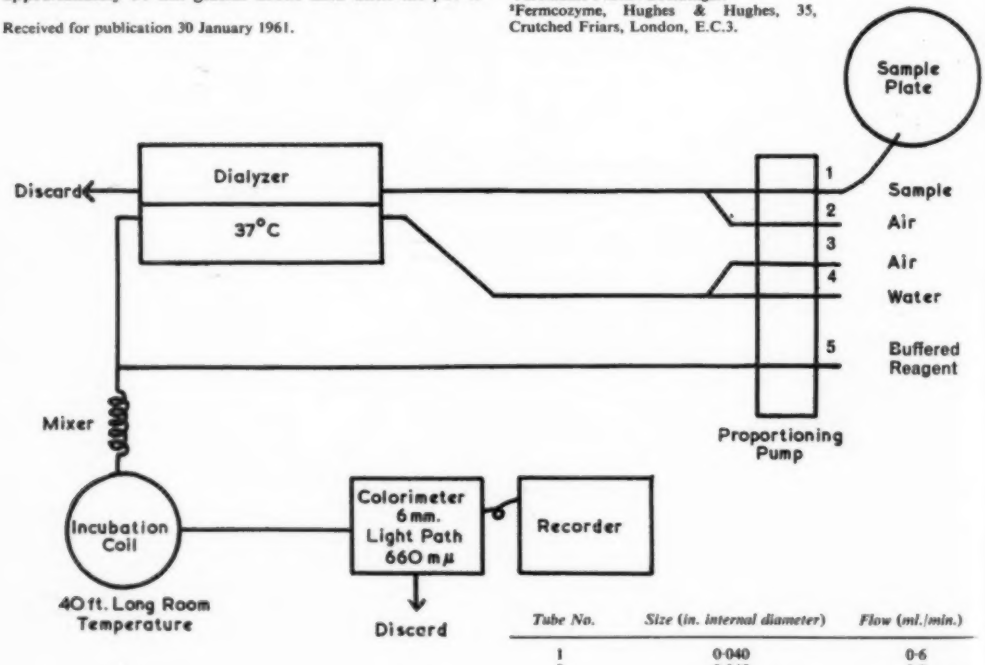
5 FORMALDEHYDE 0·1% Mix 0·25 ml. 40% formaldehyde and 1,000 units heparin in 100 ml. water. This should be prepared each week.

6 GLUCOSE STANDARDS Dissolve 2·5, 5·0, 10, 20, 30, 40, 60, and 100 mg. glucose per 100 ml. in saturated benzoic acid.

PROCEDURE FOR AUTOANALYZER

The determination is performed on the Autoanalyzer using the manifold shown on the flow chart (Fig. 1). If

[1]Peroxidase P.O.D. Boehringer.
[2]Fermcozyme, Hughes & Hughes, 35, Crutched Friars, London, E.C.3.



Tube No.	*Size (in. internal diameter)*	*Flow (ml./min.)*
1	0·040	0·6
2	0·045	0·8
3	0·045	0·8
4	0·056	1·2
5	0·090	2·9

FIG. 1. *Flow chart for Autoanalyzer.*

necessary, 2 drops of Tween 20 per litre may be added to the water in the recipient stream to ensure a good bubble pattern. Thirty determinations per hour are performed by operating the sampling module at 60 per hour and alternating samples with cups of water.

Colour development varies with the ambient temperature and when large numbers of estimations are performed standards should be run about every two hours. This may not be necessary if a constant temperature bath regulated at 25°C. is used. Colour development is poor at 37°C.

The results are read from the graph constructed from the standards using a chart reader and making allowance for dilution.

DETERMINATION OF GLUCOSE IN BLOOD AND CEREBROSPINAL FLUID

CAPILLARY BLOOD OR C.S.F. Add 0·2 ml. blood or C.S.F. to 0·8 ml. 0·1% formaldehyde solution in a sample cup. This permits of two determinations being made on each sample if required, or allows for further dilution when values above 500 mg./100 ml. are encountered; when this is unlikely 0·1 ml. blood diluted with 0·4 ml. formaldehyde suffices.

VENOUS BLOOD OR C.S.F. Heparinized or oxalated blood or C.S.F. may be preserved by the addition of 0·02 ml. 20% formaldehyde per ml. (the error due to dilution is insignificant). The blood or C.S.F. is diluted 1 in 5 with water when convenient.

Sodium fluoride in amounts sufficient to prevent loss of glucose through glycolysis was found to inhibit the colour production to a variable extent. Formaldehyde in the quantities used prevents loss of glucose for at least 24 hours even at 37°C., and does not interfere with the reaction.

The reproducibility of the technique was determined by performing 10 estimations on a single blood sample. The mean glucose level was 99·1 mg./100 ml. Standard deviation was 1·73 mg./ 100 ml. (range 97 to 102 mg./100 ml.). In a series of experiments the recovery of added glucose was 97 to 104%.

Using the chart reader, glucose values between 12·5 and 500 mg./100 ml. can be read acurately; over most of this range Beer's law is complied with.

DETERMINATION OF GLUCOSE IN URINE

The presence of inhibitory substances in urine which are not removed by dialysis makes it necessary to decolorize with activated charcoal before analysis. When amounts of glucose less than 100 mg./100 ml. are present, add 20 to 50 mg. activated charcoal to about 2 ml. urine in a sample cup, stir, and allow to settle. If larger quantities of glucose are present the urine is first decolorized and then diluted with water to give a final glucose concentration of 5 to 100 mg./100 ml.

The recovery of added glucose from urine was 93 to 112%.

A micro technique for protein concentration suitable for quantitative electrophoresis of cerebrospinal fluid

J. COLOVER[1] *From the Middlesex Hospital Medical School Institute of Clinical Research, and the Department of Neurology, Middlesex Hospital, London*

A simple apparatus has been devised for concentrating 0·5 ml. samples of cerebrospinal fluid sufficiently to enable quantitative electrophoresis for proteins on cellulose acetate to be undertaken subsequently (Colover, 1961).

It uses a principle described by Miyamoto, Anan, Arai, Sakagiski, and Nomiyama (1957) and Kohn (1959). The method is designed to avoid precipitation of proteins or drying them on a membrane, so that they may be recoverable in maximal amounts with the minimum of qualitative or quantitative change. It might thus be suitable for immunoelectrophoresis or for concentrating other biological fluids available in small quantities. Tests on one of the early prototypes of the apparatus showed that 81% to 86% of the protein, estimated by the Lowry, Rosebrough, Farr, and Randall (1951) method was recovered from it. The more recently developed chamber described here is an improvement on this earlier type as

[1]Member of the external scientific staff, Medical Research Council.
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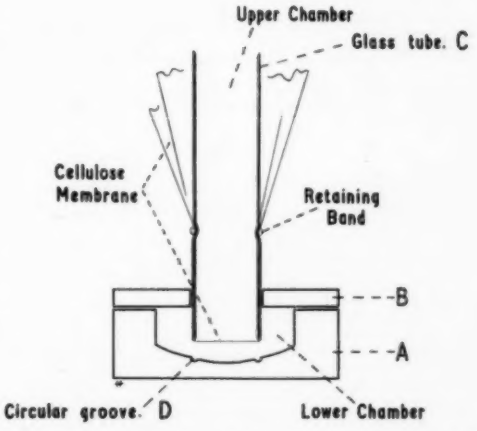

FIG. 1. *The concentration chamber (not strictly to scale.)*

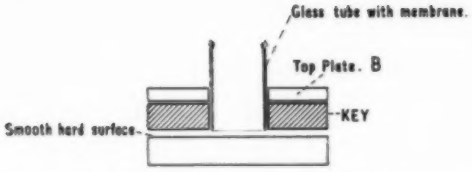

FIG. 2. *Method of adjusting projection of tube by means of a key of known thickness. After this has been done, the key is removed and the top plate, B, is placed on to the base plate, A (Fig. 1).*

can be gauged by the increased density of the bands after electrophoresis.[1]

The chamber used (Fig. 1) is made of transparent resistant plastic (Perspex) and glass and is composed of three parts. It has a square base plate (A) whose dimensions are 4 cm. × 4 cm. × 1·25 cm. This plate contains a cylindrical well 2·2 cm. in diameter, of which the side wall is 6 mm. deep. The bottom of this well is shaped as part of the surface of a sphere, and in it is cut a circular groove (D) 0·5 mm. deep. The internal diameter of the groove is 1·0 cm. and its external diameter is 1·2 cm. On the base plate rests a square top plate (B), of which the dimensions are 4·0 cm. × 4·0 cm. × 0·3 cm. similarly made of plastic material. The centre of the top plate is pierced by a circular hole 1·25 cm. in diameter. The third part of this apparatus is a glass tube (C) about 7 cm. long, having an internal diameter of 1·1 cm. and a constriction about 1·8 cm. from the lower end. This end of the tube is closed by means of a cellulose (Visking) membrane made from tubing 1 5/8 in. diameter which has been opened by cutting one side, and moistened in conductivity water. The membrane is secured to the glass tube by means of a retaining rubber band on the constriction, and should be taut. By means of a key (Fig. 2) the tube is made to project 6·25 mm. through the top plate (B).

Into the lower chamber (Fig. 1) is pipetted 0·5 ml. of cerebrospinal fluid. In the upper chamber is placed 1 ml. of 30% w/w polyethylene glycol solution in distilled water (Carbowax 20 M., G. Gurr). The two plates are kept close together by means of rubber bands and the apparatus is put in a moist chamber to prevent evaporation and kept at 4°C. for 16 hours overnight. After this period the cerebrospinal fluid has been concentrated to 5 to 20 μl., most of which lies in the circular groove. In earlier trial chambers which did not have such a groove it was found that a ring of viscid protein collected often in the base of the well opposite the edge of the glass tube. (Staining the membrane from the chamber with Ponceau S after the concentration process was completed showed little or no protein adhering to it.) To the concentrate is added 5μl. of barbitone buffer *p*H 8·6, ionic strength 0·05, and this is then transferred to a cellulose acetate membrane for electrophoresis by the method of Kohn (1957). After staining the bands with naphthalene black, the strip is scanned in an EEL scanner connected externally to a Unigalvo spot galvanometer. In this way it has been found possible to examine fluids containing as little as 15 mg. % of protein. Owing to the small amount of fluid required, the reproducibility of results obtained in replicate experiments can be investigated.

The method and results of a survey of the protein pattern of cerebrospinal fluid in multiple sclerosis and other neurological conditions will be published in detail in due course (Colover, 1961b).

It is a great pleasure to thank Professor A. Kekwick and the Clinical Research Committee of the Middlesex Hospital for facilities at the Institute of Clinical Research, and Dr. Michael Kremer for his kind support, advice, and encouragement. I have received much assistance from Mrs. P. Fry, M.Sc., and also the Courtauld Institute of Biochemistry. The prototypes of the concentration chamber were made by Mr. E. E. Dowsett. I am pleased to acknowledge grants for apparatus received from the Multiple Sclerosis Society and also a grant from the Medical Research Council.

[1]The commercial production of this apparatus is being undertaken by Messrs. Electrothermal Engineering Ltd. The design has been patented by the N.R.D.C.

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The iron method of haemoglobin estimation

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Although the estimation of haemoglobin in blood by its iron content is admittedly the most accurate of the methods available in haemoglobinometry a simple procedure for carrying this out is not described in current textbooks.

The best-known technique for determining iron, described by King and Wootton (1956), involves the ashing of a known quantity of blood in a muffle furnace, the conversion of the iron in the ash to ferrous chloride, and a final titration with titanous chloride. Such a method is not likely to commend itself, either for speed or simplicity, to the smaller laboratory. A much simpler procedure consists in the separation of iron from the haemoglobin molecule by treatment with acid, and the subsequent conversion of the liberated iron into ferric thiocyanate, which is then estimated colorimetrically. A number of such methods have been published (Berman, 1918; Brown, 1922; Wong, 1923, 1928), all based on the same principle of iron separation and conversion to thiocyanate, but differing according to the acid used to effect separation. Another point of difference is the choice of the oxidizing agent employed to ensure conversion of the iron to the ferric state: without such oxidation a large proportion of the iron may remain in the ferrous state and would thus escape detection when treated with thiocyanate. A critical study of the published methods, though confirming their accuracy, has shown them to be unnecessarily elaborate, and the following procedure has been devised which enables a result to be obtained in about 15 minutes, with an error not exceeding 2%, when samples of blood of known content supplied by the Medical Research Council were analysed.

REAGENTS

1 H_2SO_4, A.R.

2 Saturated potassium chlorate prepared by dissolving 10 g. in 100 ml. distilled water with the aid of heat, allowing to cool and filtering.

3 3N KCNS (29·7%)

4 10% sodium tungstate

5 Standard iron solution prepared as follows:—Ferrous ammonium sulphate, 0·7 g., is dissolved in 10 ml. distilled water. Then 5 ml. concentrated sulphuric acid is added and the mixture slightly warmed. N/10 potassium permanganate is added until oxidation of iron is complete, as indicated by a persisting permanganate tinge: about 15 ml. is required for this. Distilled water is then added up to one litre. This solution contains 0·1 mg. iron in 1 ml.

PROCEDURE

1 To a test tube containing 2 ml. distilled water add 0·1 ml. blood, 0·4 ml. saturated potassium chlorate, and 0·4 ml. concentrated sulphuric acid.

2 Mix and place in a boiling water bath for five minutes.

3 Cool, add 0·4 ml. 10% sodium tungstate and mix, and transfer to a 10 ml. cylinder and make up to 10 ml. with distilled water.

4 Mix, stand for two minutes, and centrifuge.

5 Pour off the supernatant, pipette off 8 ml. and add 2 ml. 3N KCNS and read the optical density, using a green filter.

6 For the standard, pipette 0·4 ml. into a test tube and add 7 ml. distilled water, 0·6 ml. saturated potassium chlorate, and 2 ml. KCNS.

7 If extinctions of standard and unknown are a and b respectively

$$\text{then } \frac{a}{b} = \frac{\text{iron content of standard}}{\text{iron content of unknown}}$$

$$\therefore \text{Fe content of unknown} = \frac{b}{a} \times 0{\cdot}04 \text{ mg.}$$

$$\text{Haemoglobin of unknown} = \frac{b}{a} \times 0{\cdot}04 \times 300 \text{ mg.}$$

$$(\text{assuming Fe} = 0{\cdot}334\% \text{ of Hb})$$

$$= 12\frac{b}{a} \text{ mg.}$$

Since unknown tube contained iron from 0·08 ml. blood, then haemoglobin content of blood $= \frac{b}{a} \times 12 \times \frac{10}{8} = 15\frac{b}{a}$ g%.

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Book reviews

MODERN SCIENTIFIC ASPECTS OF NEUROLOGY. Edited by John N. Cumings. (Pp. 360; 89 figures + 13 colour plates. 65*s*.) London: Edward Arnold. 1960.

During the last decade progress in the science of neurology has been spectacular, especially in the fields of ultrastructure and of biochemistry. This volume is designed to bring the practising neurologist right up to date on these and other fundamental aspects of his subject. It should succeed admirably in its purpose and the pathologist who is concerned in the study of neurological disease will find it a gold mine of information.

The chapter on electronmicroscopy by Professor F. S. Sjöstrand helps one to appreciate the importance of membrane interfaces in ionic exchange while that of Dr. J. B. Finean on the *x*-ray diffraction analysis of nerve myelin gives an important and vivid insight into the molecular pattern of myelin. Because the primary demyelinating diseases and, in particular, multiple sclerosis, constitute the greatest neurological cause of incapacity in young adults, interest today largely centres on myelin. It is understandable therefore that half of the chapters in this book, including an important one contributed by Professor Cumings himself, are orientated around this theme and the related lipidoses and, if in recent years they have accounted for a plethora of publications, it is but a measure of the amount of work which has been undertaken, and of the importance which is attached to their study. The chapters on the histochemistry of the demyelinating diseases by Professor Seitelberger and on the lipidoses by Dr. P. B. Diezel will prove helpful to pathologists. Dr. Larus Einarson discusses the role of the nucleic acids in the metabolism of the neurone, while Professor R. H. S. Thompson considers the esterases of the nervous system and the condition of anticholinesterase intoxication. Though much has been written in recent years about nerve endings in striated muscle, the excellent and lucid review of the subject by Dr. C. Cöers will be particularly welcome.

This is an agreeable production and the illustrations are as well reproduced as they are selected.

P. M. DANIEL

LEUKAEMIA: RESEARCH AND CLINICAL PRACTICE. By F. G. J. Hayhoe. (Pp. viii + 335; 12 colour plates, 196 black-and-white figures. 80*s*.) London: J. & A. Churchill. 1960.

Among advances in the field of haematology during the past decade, the study and treatment of leukaemia has been prominent. Yet results have been frustrating and we are still denied the 'break through' which surely must come, permitting the replacement of palliative by rational therapy based on aetiology rather than on sequelae: the use of the all-embracing term leukaemia, related to the result rather than the cause, is one indication of this frustration. Unhappily, attempts at classification are bedevilled by the paradox of disease processes, having nothing in common save the generic term leukaemia, being confused with closely allied conditions which are never 'leukaemic'.

During this period of obscurity it is well to have the present situation viewed in perspective and two substantial texts are currently available for the clinician. In 1958 'Leukemia' emanated from the Boston school and has now been followed in 1960 by 'Leukaemia' presenting the English viewpoint. Chauvinism or no, the latter is easily readable and provides, in Dr. Hayhoe's words, ' guide to the maze of published work which separates the tangled lines of progress in reasearch'.

The book is in four main sections. A preliminary historical account (incidentally this makes pleasant reading) is followed by a chapter on classification. In practice, classifications suffer from being too comprehensive, for while each worker tries to include every facet, with the best will in the world one is still left with a group of 'variable, multiple or uncertain origin' and at least one reader feels that the fewer classifications there are the better. Particularly difficult to assign are what Dr. Hayhoe refers to as the 'paraleukaemic' states but which others include among the myeloproliferative dyscrasias. The latter term is convenient—very helpful to the worker—as long as one does not necessarily imply that these disorders share a common aetiology: but study of the dynamics of some of them raises the doubt as to whether the reaction is indeed proliferative. The chapter on statistics is helpful and the somewhat philosophical review of experimental studies of aetiology is concise; the author rightly acknowledges that data from animal leukaemias cannot be held to apply directly to the disease in man.

The second section, discussing the leukaemic cell, is excellent. It is Dr. Hayhoe's major contribution that morphology in leukaemia is given a new look. The standard morphological criteria have stood the test of time but are no longer wholly adequate: perhaps refined cytological and histochemical techniques may also prove to be insufficient, but this approach is well worthwhile if it permits more workable divisions of these diseases, linked with prognosis or susceptibility to therapy; the P.A.S. and leucocyte alkaline phosphatase reactions must now be utilized as routine differentiating techniques. Colour plates of microscopic morphology, so often the publisher's pride, are frequently disappointing, especially those attempting to reproduce Romanowsky stains. It is noteworthy that those selected for inclusion in this volume are, in the main, successful. Their publication is entirely justified for they portray the histochemical tinctorial reactions upon which Dr. Hayhoe places diagnostic emphasis. Much has yet to be learned about culture techniques *in vitro* and these have had disappoi t-

ing results, but it may be possible in the future to link them with histochemical investigations.

The third section is the most useful to the haematologist for it provides a résumé of current therapeutic trends. Inevitably this must date rapidly, but this is a standard risk for all major textbooks and Dr. Hayhoe's approach, by discussing the underlying motivation, provides a sound stem upon which newer therapy may be grafted.

The fourth section refers to clinical aspects. Reading through these chapters reinforces the view that any one classification can never adequately embrace this group of pathological states, but Dr. Hayhoe makes a sporting attempt in his final figure where every condition is brought into one major scheme.

This book can very thoroughly be recommended for all haematologists. Of particular value is the very adequate bibliography.

Dr. Hayhoe ends his book with a brief attempt at definition. '. . . the leukaemias are proliferative diseases falling within the general category of neoplasia in its widest sense, but very possibly due to an externally determined imbalance between the forces controlling leucopoiesis, although in some cases the proliferation may become autonomous'. It will be interesting to see how this definition stands when, in due course, this volume progresses to a second edition. It might be helpful on such an occasion for the author to summarize each chapter, giving his own views on the material analysed and presented. This would provide a more personal touch to this first-class review of a very difficult subject.

J. L. STAFFORD

A SYMPOSIUM ON *p*H AND BLOOD GAS MEASUREMENT: METHODS AND INTERPRETATION. Edited by Ronald F. Woolmer, assisted by Joy Parkinson. (Pp. viii + 210; 48 figures. 30*s*.) London: J. & A. Churchill. 1959.

This is a record of the symposium held under the auspices of the Ciba Foundation in December 1958, and includes the nine papers given and the discussions which followed. The subjects were methods of estimating blood *p*H and deriving pCO_2 by the Henderson-Hasselbalch equation; the more recent method of deriving pCO_2 from estimations of the *p*H of plasma as collected and after equilibration with gas mixtures of known pCO_2 (thereby dispensing with the need to estimate total plasma CO_2); the determination of mixed venous and arterial pCO_2 by rebreathing techniques; the use of blood O_2 and CO_2 electrodes; and the clinical significance of the values obtained.

The contributors are all experts in their particular fields, and the book assembles a great deal of useful information which is widely scattered in the journals. It is concerned primarily with respiratory disorders of acid-base balance and with the problems of the anaesthetist, but there is much of interest to the clinical pathologist who investigates acid-base disturbances of any sort.

G. K. MCGOWAN

A Ciba Foundation Symposium on CONGENITAL MALFORMATIONS. Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. xii + 308; 91 figures. 45*s*.) London: J. &. A. Churchill. 1960.

This volume is very welcome with the increasing interest in the nature and incidence of congenital malformations. A chapter on the probable incidence of malformations in the general population is followed by a discussion of genetical causes, and an account of chromosome abnormalities recently discovered. Environmental factors receive due consideration and are succeeded by a series of accounts of experimental procedures. Malformations have been induced by a wide variety of substances including antimetabolites, such as 6-mercaptopurine, and the antibiotic actinomycin D. Many general pathologists will find this work fascinating since probably few realize its extent. Not only have malformations been induced by these chemicals, but there is in addition evidence that other substances can modify their effect. It therefore cannot be long before this experimental work leads to new information of great practical value in clinical medicine.

The standard of production is well up to the high level which we have come to expect from other monographs of symposia held by the Ciba Foundation. The discussions are of the greatest interest and value and reflect much credit on the editor.

G. J. CUNNINGHAM

CANCER PROGRESS 1960. Edited by Ronald W. Raven. (Pp. XV + 242 + Index; 45 figures. 60*s*.) London: Butterworth. 1960.

Excellently written throughout, this progress volume contains brief but interesting accounts of current research in malignant disease and many valuable critical reviews of topicl subjects, all well garnished by recent references given with full titles. Of particular interest to clinical pathologists are the articles on tumour virology, bone marrow replacement therapy, circulating free cancer cells, mammary sarcomas, industrial aspects of respiratory tract carcinoma, clinical and metabolic aspects of carcinoid tumours, and chemotherapy of malignant disease including lymphoma and leukaemia.

The real purpose of this book, however, is to acquaint workers in clinical and non-clinical fields with modern trends in cancer diagnosis, treatment, control and research, including social and statistical studies. To achieve this aim the editor, in addition to his original team of experts in most aspects of the cancer problem, has elicited the aid of several internationally acknowledged authorities as advisory editors. In attaining the set objective so successfully the editor and his collaborators are to be highly commended.

R. WINSTON EVANS

CHOLERA: ITS PATHOLOGY AND PATHOGENESIS. By S. N. De. (Pp. xi + 141; 50 figures. 25*s*.) Edinburgh: Oliver and Boyd. 1961.

The first three chapters of De's book review the history of cholera and of investigations on it both in Europe and in India. Though commonly called 'asiatic', there is some doubt as to the real origin of the disease and considerable doubt whether the 1817 pandemic really represented its first invasion of Europe.The epidemiology of present-day cholera in Calcutta is then described, with several charts to illustrate seasonal variation.

The pathology of the disease is described and illustrated, and this is followed by an account of De's own work, some hitherto unpublished, on the experimental reproduction of the disease in rabbits. De believes that an exotoxin plays an important part. The last four chapters revert to the review style and describe the process of infection and the factors thought to lead to the various clinical facets of the disease.

Altogether the book makes interesting reading; for further search in the literature, each chapter is provided with a long list of references. The illustrations include several good portraits of the pioneers of cholera study and many that reveal the major hygienic problems of Calcutta even now.

R. E. O. WILLIAMS

BASIC PROBLEMS IN NEOPLASTIC DISEASE

Columbia University College of Physicians and Surgeons is sponsoring a three-day symposium on 'Basic Problems in Neoplastic Disease', to be held on 12, 13, and 14 March 1962.

Sessions will be held on 'Nucleic Acid Structure and Synthesis', 'Viral and Genetic Studies', 'Protein Synthesis', 'Antibody Structure and Function' and on clinical aspects of the biochemistry, pathological-physiology, morphology, and therapy of cancer.

The symposium is open without fee. For details concerning the meeting, including application to attend sessions, write to the Institute for Cancer Research, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York.

Broadsheets prepared by the Association of Clinical Pathologists

The following broadsheets (new series) are published by the Association of Clinical Pathologists. They may be obtained from **Dr. R. B. H. Tierney, Pathological Laboratory, Boutport Street, Barnstaple, N. Devon.** The prices include postage, but air mail will be charged extra.

3 The Detection of Barbiturates in Blood, Cerebrospinal Fluid, Urine, and Stomach Contents. 1953. L. C. NICKOLLS. 1s.

4 The Estimation of Carbon Monoxide in Blood. 1953. D. A. STANLEY. 1s.

5 The Identification of Reducing Substances in Urine by Partition Chromatography on Paper. 1953. G. B. MANNING. 1s.

6 The Paul-Bunnell Test. 1954. R. H. A. SWAIN. 1s.

7 The Papanicolaou Technique for the Detection of Malignant Cells in Sputum. 1955. F. HAMPSON. 1s.

10 Mycological Techniques: (1) Collection of Specimens. 1956. R. W. RIDDELL. 1s.

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12 Techniques for Demonstrating L.E. Cells. 1956. J. V. DACIE and L. S. SACKER. 1s.

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16 Preservation of Pathological Museum Specimens. 1957. L. W. PROGER. 1s.

17 Cultural Diagnosis of Whooping-cough. 1957. B. W. LACEY. 1s.

18 The Rose-Waaler Test. 1957. C. L. GREENBURY. 1s.

19 The Laboratory Diagnosis of Fibrinogen Deficiency. 1958. R. M. HARDISTY. 1s.

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32 Detection of Resistance to Streptomycin, P.A.S., and Isoniazid in Tubercle Bacilli. 1961. R. CRUICKSHANK and S. M. STEWART. 2s.

34 Titration of Antistreptolysin O. 1961. H. GOOLDER and R. E. O. WILLIAMS. 2s.

35 The Estimation of Faecal 'Urobilinogen.' 1961. C. H. GRAY. 2s.

J. clin. Path. (1961), **14**, 565

The histological diagnosis of toxoplasmic lymphadenitis

A. G. STANSFELD

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SYNOPSIS The commonest presenting sign of acquired toxoplasmosis in man is enlargement of superficial lymph nodes. The persistence of the nodes may lead to a suspicion of malignant lymphoma and the diagnosis then hinges on the lymph node biopsy.

Three cases of toxoplasmic lymphadenitis are described in each of which the diagnosis was unsuspected clinically. The chance discovery of a toxoplasma cyst in the lymph node section of Case 1 led to the correct diagnosis, after an initial diagnosis of Hodgkin's disease had been made. In the other two, strikingly similar histological changes in the lymph node biopsies suggested the diagnosis, which was confirmed serologically in each case.

The histological changes are described and the clinical and pathological aspects of toxoplasmic lymphadenitis are briefly reviewed, with special reference to the differentiation from malignant lymphomatous conditions and to the specificity of the histological picture. It is concluded that the histology is, in many instances, sufficiently distinctive for a tentative diagnosis of toxoplasmic lymphadenitis to be made on the lymph node biopsy. The diagnosis should always be confirmed by isolation of the parasite or by serological tests. It is exceedingly rare for toxoplasma cysts to be found in lymph nodes and only one previous observation of this kind has been reported.

Over the past 20 years it has become evident that human infection with *Toxoplasma gondii* is almost world-wide in distribution and that this remarkably successful parasite has, as alternative hosts, a wide range of mammals, birds, and even reptiles. In man, infection is not nearly as rare as was formerly thought. Not unnaturally, the first cases of acquired toxoplasmosis to be diagnosed were all examples of the severe, acute form of the disease, ending fatally. This variety is undoubtedly rare, but within the past decade a number of reports have been published of a much commoner manifestation of acquired toxoplasmosis, characterized by enlargement of superficial lymph nodes, often without other conspicuous symptoms.

The importance of toxoplasmic lymphadenitis lies in its differentiation from much more serious conditions, especially Hodgkin's disease and lymphosarcoma, for which it may be mistaken on both clinical and pathological grounds. Writers on the subject have expressed different opinions about the specificity of the histological changes in the lymph nodes in toxoplasmosis. Nevertheless there is in many of the accounts a striking measure of agreement about the principal histological features, allowing for differences in terminology.

The three cases reported here serve to emphasize the relative frequency of the condition, the constancy of the histological changes in the lymph nodes, and the importance of lymph node biopsy in arriving at the diagnosis.

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CASE REPORTS

CASE 1 A.M., a married woman of 30, was referred to St. Bartholomew's Hospital on 13 October 1960 for radiotherapy after a diagnosis of Hodgkin's disease had been made at another hospital on a cervical lymph node biopsy.

For six to seven weeks she had noticed swellings in the neck but felt otherwise quite well. Her appetite was good and weight steady.

Physical examination showed a well nourished woman. Two enlarged lymph nodes, each nearly 2 cm. in diameter, were palpable in the neck: one on the right side, at the upper end of the posterior triangle; the other, in the left supraclavicular fossa, lying just below the biopsy incision. There were no enlarged nodes in axillae or groins and neither liver nor spleen were felt on abdominal examination.

The results of laboratory investigations were as

follows:—E.S.R. 7 mm. in the first hour, haemoglobin 86% (12·7 g. haemoglobin / 100 ml.) W.B.C. 5,000 per c.mm. (polymorph neutrophils 70%, eosinophils 2%, lymphocytes 20%, monocytes 8%), platelets 176,000 per c.mm. A radiograph of the chest did not show any abnormality.

The lymph node biopsy section was re-examined at St. Bartholomew's at the start of radiotherapy and the changes seen were then interpreted as reactive rather than neoplastic. In the course of this examination a single cyst of toxoplasma was encountered in the section and a diagnosis of toxoplasmic lymphadenitis was made on the strength of this discovery. The diagnosis was confirmed a few days later by a positive dye test at a titre of 1 in 1,000.

The patient was subsequently given a 14-day course of triple sulphonamide and pyrimethamine and, three weeks after its conclusion, examination failed to disclose any palpable lymph nodes in the neck or elsewhere. She has remained well, but it was later reported that her son, aged 2 years, had an asymptomatic enlargement of cervical lymph nodes.

CASE 2 D.L., a married woman of 40, was referred to another hospital on 10 November 1960 by her doctor for the investigation of enlarged cervical lymph nodes.

Four months previously she had noticed a swelling behind the left ear, but this had disappeared spontaneously. A month later, a swelling appeared behind the left sternomastoid and grew steadily in size, while a second smaller swelling appeared on the right side too. She had not had a sore throat. The swelling was slightly tender at first but was not painful, though she experienced some difficulty in turning her head to the left. The swellings had persisted since. For a period of about three months she had also complained of indigestion and epigastric discomfort after food. She had also noticed tiredness and breathlessness but had no cough and her weight was steady.

Physical examination showed a well nourished woman. There were two enlarged, fairly soft nodes on the left side of the neck and one on the right, while two similar nodes were also found in the right axilla. The liver and spleen were not felt. On the basis of these findings and the history it was thought most likely that she had some form of 'lymphomatous neoplasia'.

The chest radiograph was normal and Wassermann, Kahn, and Price's precipitation reactions gave negative results. A blood leucocyte count before admission had shown a total of 5,000 cells per c.mm. (polymorph neutrophils 35%, lymphocytes 60%, and monocytes 5%). The E.S.R. varied between 18 and 25 mm. in one hour and the haemoglobin was 90% (13·3 g. haemoglobin/100 ml.). A relative lymphocytosis persisted for several weeks, though the total leucocyte count remained low. No atypical cells were seen in blood smears. A Paul-Bunnell screening test performed at the beginning of December was negative.

On 16 November biopsy of a cervical lymph node was carried out. The node, which measured 2·3 × 1·8 × 0·5 cm., showed a hard, white cut surface with brown streaks. The pathologist reported that the histological picture corresponded with that of lympho-histiocytic medullary reticulosis (Robb-Smith, 1947), which he regarded as a reactive response of relatively non-specific type. The section was referred to me for my opinion and, in view of the remarkable similarity of the histological appearances to those of Case 1, a diagnosis of toxoplasmic lymphadenitis was suggested. Toxoplasma cysts were not found in the original section or in sections subsequently examined but the diagnosis was confirmed serologically. The patient's serum, taken on 3 December, gave a positive dye test at a titre of 1 in 4,096 and the complement-fixation test was positive at 1 in 32.

No treatment was given and on 21 December the lymph nodes were reported to be subsiding.

CASE 3 R.A.L-N., an advertising representative, aged 19, was referred by his own doctor to St. Bartholomew's Hospital on 14 November 1960 with a swelling beneath his chin which was thought to be a thyroglossal cyst.

He had first noticed the swelling, which was well to the left of the midline, two to three months previously and at that time another swelling had appeared on the right side of the neck also. The latter had disappeared following a course of sulphonamide treatment. He had had no pain, sore throat, or cough and his weight was steady. Tonsillectomy had been performed at the age of 7.

Physical examination showed a young man of healthy appearance with a visible swelling beneath the chin on the left side. On palpation, a fairly soft, doubtfully fluctuant swelling, 1 in. × ½ in., could be felt in this situation. A few soft nodes were palpable in the right axilla. The liver and spleen could not be felt.

He was kept under observation and, two months later, since the swelling showed no diminution in size, he was admitted to hospital for biopsy. Examination now showed a small palpable tonsillar lymph node on the right side, as well as the swelling on the left. Laboratory tests showed: Haemoglobin 102% (15·1 g. haemoglobin/100 ml.), W.B.C. 6,000 per c.mm. (polymorph neutrophils 65%, eosinophils 2%, lymphocytes 24%, monocytes 9%).

On 13 January 1961 the swelling beneath the chin was excised and proved to be an enlarged lymph node, measuring 3·0 × 1·6 × 0·3 cm. The consistency was firm and the cut surface of the node showed a faintly discernible follicular pattern. The histological appearances were sufficiently similar to those of Cases 1 and 2 as at once to suggest a diagnosis of toxoplasmic lymphadenitis. Cysts were not found but the diagnosis of toxoplasmosis was confirmed serologically. The patient's serum, taken on 18 January, gave a positive dye test at a titre of 1 in 2,048 and the complement-fixation test was positive at 1 in 64. A course of treatment with triple sulphonamide and pyrimethamine was given subsequently and when last seen the patient was symptom free.

HISTOLOGY

The diagnosis of toxoplasmosis was made in Case 1 by the chance discovery of a toxoplasma cyst in the lymph node section. This section also showed rather distinctive reactive changes, and it was the finding of

very similar appearances in the lymph node biopsy sections that led to the diagnosis in Cases 2 and 3. The following description of the histological changes is based upon all three cases.

The lymph node in each instance was markedly enlarged, but apart from this there was nothing remarkable about the macroscopic appearance.

On microscopical examination, the essential features are those of a subacute to chronic lymphadenitis with well-marked periadenitis. A striking degree of lymphoid hyperplasia is present which is, no doubt, mainly responsible for the lymph node enlargement. Many of the follicles are large and irregular in outline with active germinal centres (Fig. 1). These are, however, often less clearly defined than is commonly the case in reactive hyperplasia, and proliferating lymphoblasts sometimes mingle with mature lymphocytes, giving the impression of an infiltrative lesion. There are numerous mitoses of regular pattern in the germinal centres and a notable feature is the presence of many freshly necrotic cells, so that the centres are littered with karyorrhectic particles of nuclear débris, some of which have been engulfed by macrophages (Figs. 2 and 3).

Scattered through the pulp of the node, and sometimes invading the follicles, there are small clusters of very conspicuous, pale-staining histiocytes with vesicular nuclei and copious, somewhat eosinophilic cytoplasm (Figs. 3, 4, and 5). These cells resemble the epithelioid cells of tuberculosis, but multinucleate cells are scarce and none simulate Langhans giant cells. Moreover caseation is absent. The cell clusters are smaller and generally less sharply defined than the focal collections of similar cells seen in sarcoidosis. A further contrast with sarcoidosis is seen in the absence of reticulin in relation to these cells in a silver impregnation preparation (Fig. 6).

The presence of these histiocyte clusters is perhaps the most distinctive feature of the histological picture in all three cases. They are not regularly distributed, but are chiefly seen towards the periphery of the node which presents a spotted appearance on low-power examination (Fig. 4). They appear to be engaged in the phagocytosis of nuclear débris liberated by the piecemeal necrosis of cells in the follicles. Infiltration of the follicles by histiocytes is certainly one reason for the blurring of the follicular outline.

Almost as distinctive a feature as the histiocyte clusters is the dense packing of many of the lymph sinuses with free macrophages (Fig. 7). In sections of each of the three lymph nodes some of the sinuses are so completely filled that they would not be distinguishable, in the haematoxylin- and eosin-stained section, from the dense pulp of the node, but for the smaller size and stronger basophilia of the lymphocytes in the latter. Both peripheral and central sinuses may be affected, but the density of the cellular infiltration varies and, in places, discrete cells are seen against a background of eosinophilic coagulum.

High-power examination reveals a mixed population of cells in the lymph sinuses, including a scattering of neutrophil polymorphs and some large macrophages laden with cellular débris. The predominant cell, however, is a small macrophage with many of the characters of a blood monocyte. These 'monocytoid' cells have relatively large, often contorted, nuclei and moderately strongly staining, amphophilic cytoplasm (Fig. 8). They are quite distinct from the large epithelioid histiocytes described above (Fig. 7), and also from the proliferated littoral cells commonly found in reactive conditions.

Lastly, there is a marked increase of plasma cells throughout the pulp of the node, especially in the medullary cords and around the small blood vessels. The node capsule is thickened and infiltrated with cells and this infiltration, which includes many plasma cells, extends out into the surrounding adipose tissue (Fig. 9). Eosinophil leucocytes are scarce or absent in all three biopsies.

Numerous sections, from all three cases, were searched for organisms and Giemsa's method was used as well as haematoxylin and eosin staining. Apart from the cyst originally discovered in Case 1, no other definite organisms were found. The cyst, which does not appear to be intracellular, is located at the margin of a lymph follicle. It is oval in shape, perhaps due to compression by surrounding cells, and shows a definite external limiting membrane which stains with eosin. Inside the cyst, a large number of very small toxoplasms can be distinctly seen (Fig. 10).

DISCUSSION

Up to a decade ago, the only known cases of acquired toxoplasmosis in man took the form either of a severe illness resembling Rocky Mountain spotted fever (Pinkerton and Weinman, 1940; Pinkerton and Henderson, 1941), or of an almost equally fatal disease characterized mainly by encephalitis (Sabin, 1941). It was realized that there was a marked discrepancy between the rarity of these adult cases and the comparative frequency of congenital toxoplasmosis in babies. With these facts in mind, Siim in Denmark and Magnusson in Sweden began to seek for evidence of a milder form of the disease in adults. In this they were greatly assisted by the newly introduced cytoplasm-modifying antibody reaction (dye test) of Sabin and Feldman (1948).

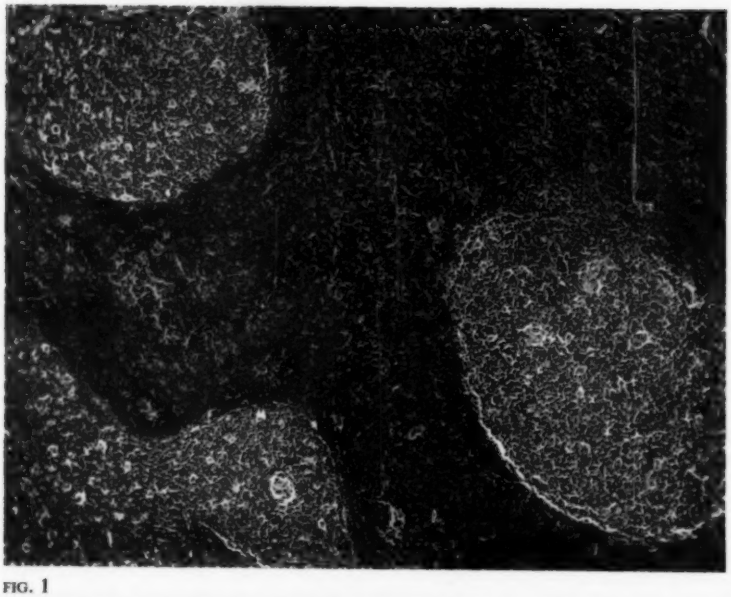


FIG. 1

FIG. 1. *Enlarged and irregular lymph follicles (Case 3). Haematoxylin and eosin × 65.*

FIG. 2. *Germinal centre of follicle showing mitroses and necrotic cells (Case 1). Haematoxylin and eosin × 400.*

FIG. 3. *Margin of enlarged follicle showing nuclear fragments and invading epithelioid histiocytes (Case 1). Haematoxylin and eosin × 135.*

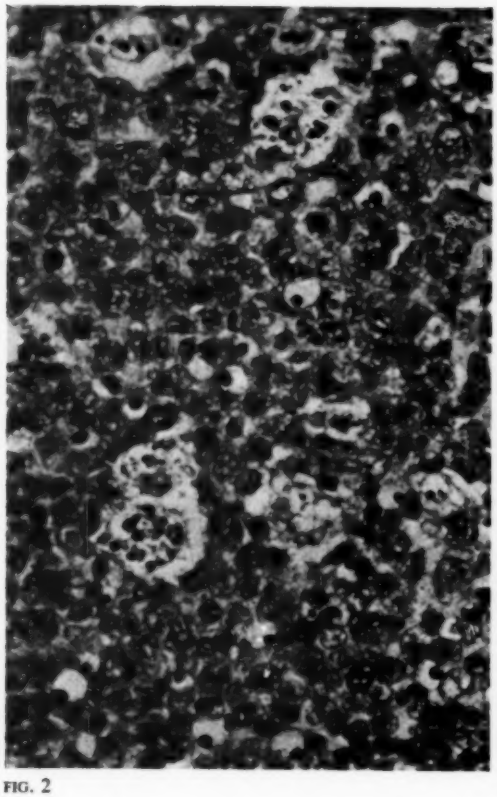
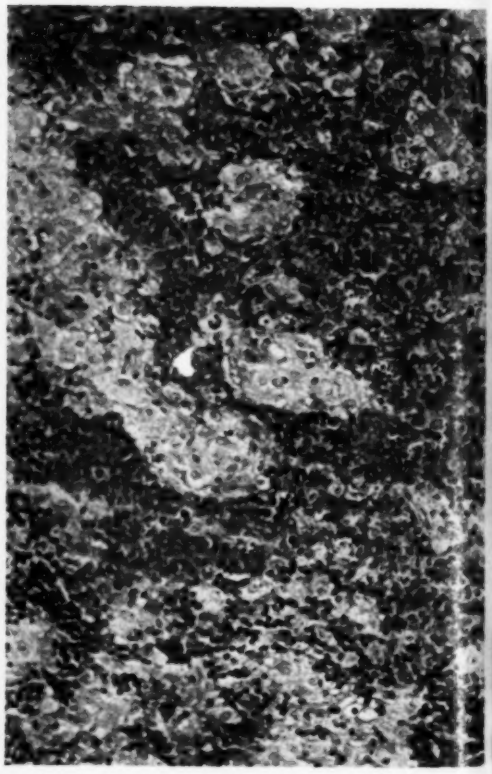


FIG. 2



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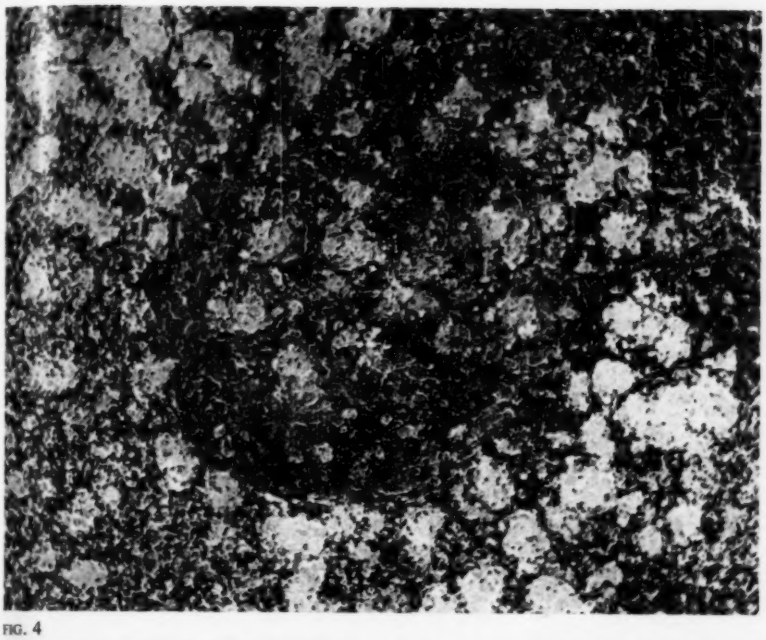

FIG. 4

FIG. 4. *Clusters of epithelioid histiocytes at periphery of cortex (Case 3). Haematoxylin and eosin × 85.*

FIG. 5. *Detail of epithelioid histiocyte clusters (Case 3). Haematoxylin and eosin × 400.*

FIG. 6. *Epithelioid histiocyte clusters showing absence of reticulin formation (Case 3). Gordon and Sweet's method × 350.*

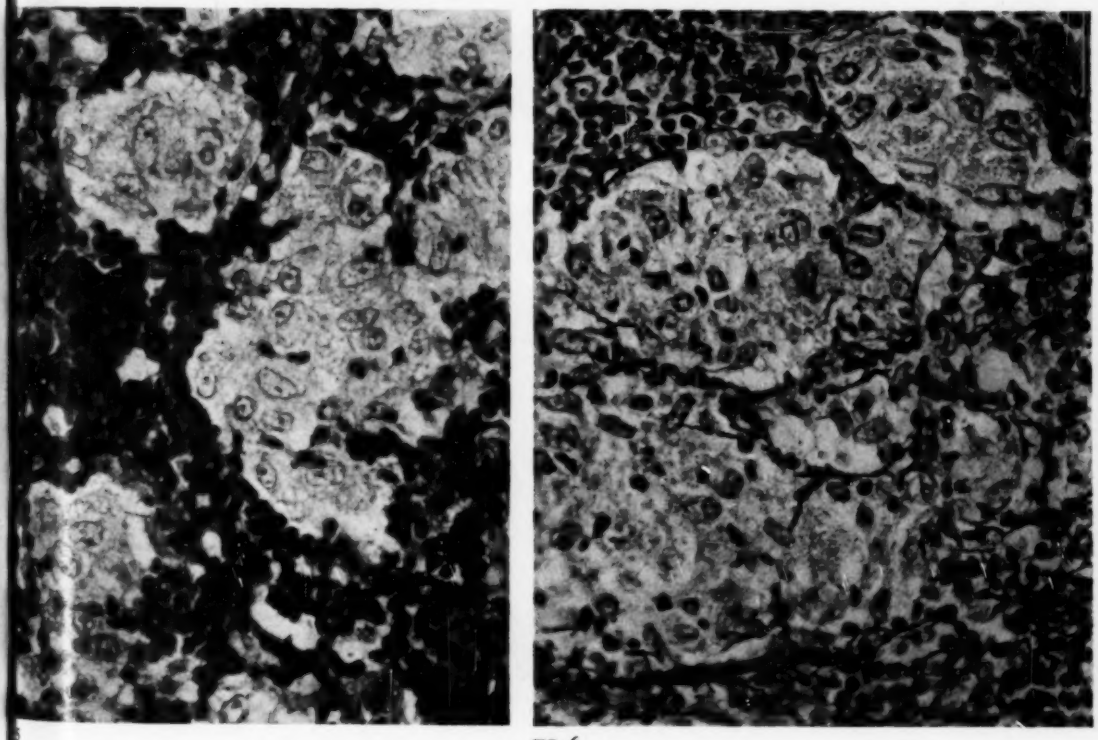

FIG. 6

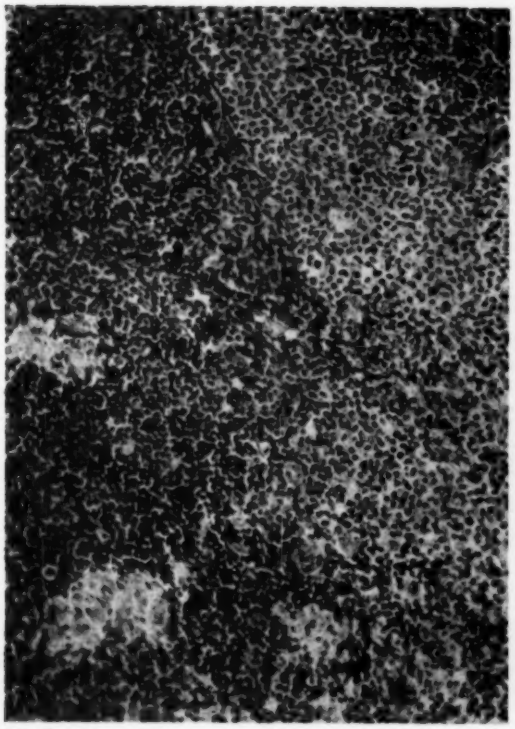

FIG. 7

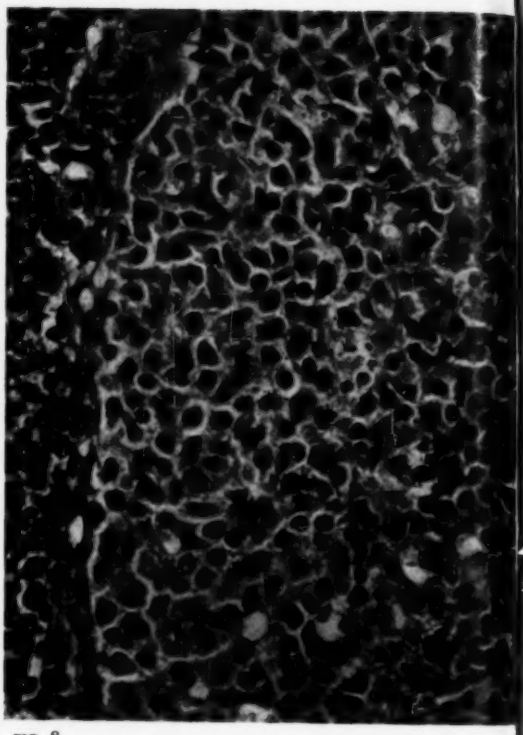

FIG. 8

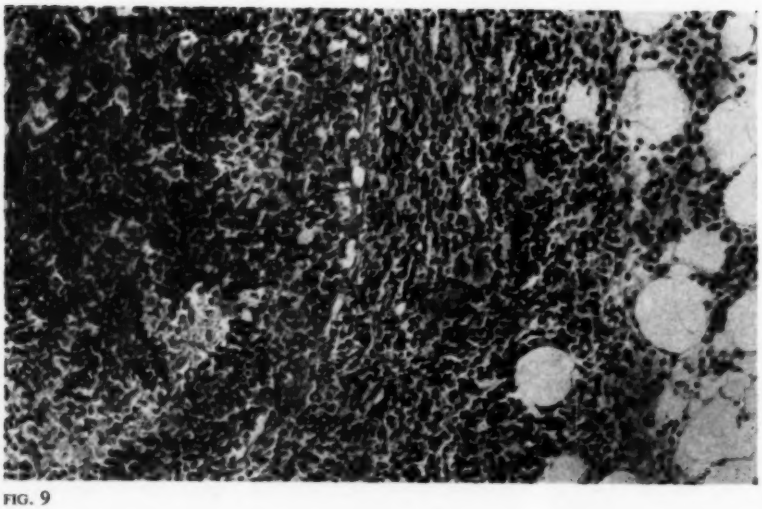

FIG. 9

FIG. 7. *Dense packing of lymph sinuses with monocytoid macrophages. Note epithelioid histiocytes at upper left (Case 2). Haematoxylin and eosin × 125.*

FIG. 8. *Details of cells filling lymph sinus (Case 2). Haematoxylin and eosin × 400.*

FIG. 9. *Periphery of node showing capsular and extracapsular infiltration by cells, including many plasma cells (Case 2). Haematoxylin and eosin × 135.*

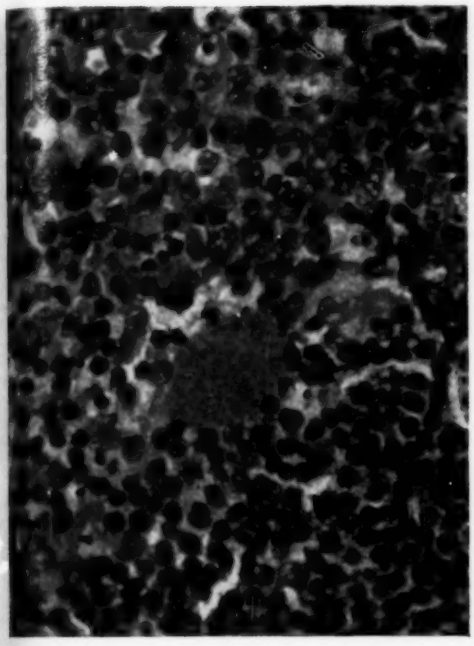

FIG. 10. *Toxoplasma cyst at margin of follicle (Case 1). Haematoxylin and eosin × 500.*

The first published account of glandular toxoplasmosis was that of Gard and Magnusson (1951) who described three adult patients with the disease. In the succeeding year or so, several reports of similar cases appeared from Scandinavia (Siim, 1951 and 1952; Wahlgren, 1951; Landau, 1951; von Zeipel and Linder, 1951; Magnusson, 1951; Bang, 1953), followed by reports from the United States (Armstrong and MacMurray, 1953; Stanton and Pinkerton, 1953). The first account of glandular toxoplasmosis in this country was that of Skipper, Beverley, and Beattie (1954) and, since that time, there have been a number of further publications on the subject from many parts of the world.

The number of these reports and the fact that I encountered three cases in the space of a few weeks suggest that the condition is far from rare. Indeed, the frequency of positive serological reactions for toxoplasma among series of apparently healthy adults indicates that the cases presenting clinically still form only a small proportion of the total number of infected individuals. It is probable that more cases will be diagnosed in the future, with increasing awareness of the condition and the greater use of lymph node biopsy as a diagnostic procedure.

The clinical aspects of toxoplasmic lymphadenitis have been recently reviewed by Beverley and Beattie (1958) and by Remington, Jacobs, and Kaufman (1960). The disease is commonest in older children and young adults. Some European authors (Bang, 1953; Piringer-Kuchinka, Martin, and Thalhammer, 1958; Saxén and Saxén, 1959) have observed a preponderance of female patients, but in reports from this country the sexes have been almost equally affected. Saxén and Saxén (1959) noted a seasonal prevalence of the disease in the winter months.

For a variety of reasons little is known of the epidemiology of acquired toxoplasmosis and the only cases in which the mode of infection has been definitely established have been instances of laboratory infection, where workers handling the organism have pricked themselves with infected hypodermic needles (Gard and Magnusson, 1951; Ström, 1951; Beverley, Skipper, and Marshall, 1955; Kayhoe, Jacobs, Beye, and McCullough, 1957). In the naturally occurring disease, it has been suggested that infection may occur most commonly by the respiratory route, with primary involvement of the nasopharyngeal lymphoid tissue (Beattie, 1957; Lainson, 1958). This would agree with the observation that the cervical lymph nodes are commonly affected and there is not infrequently a sore throat at the onset of symptoms (Beverley and Beattie, 1958). Commonly the lymphadenopathy extends to involve other nodes, especially the axillary and inguinal groups. Enlarged mediastinal nodes have been observed radiographically on several occasions and it has been claimed that the retroperitoneal and mesenteric nodes may be affected (Beverley and Beattie, 1958; Remington *et al.*, 1960).

The chief importance attaching to this generally mild disease is that it may be mistaken for disorders of a more serious nature. Clinically, one of the characteristics which may lead to a suspicion of malignancy is the persistence of the lymph node enlargement, often for several months and sometimes for a year or longer. Some patients complain of marked fatigue and general debility, but loss of weight is not a feature. If there is fever at the outset, infectious mononucleosis may be suspected and the presence of a lymphocytosis with atypical lymphocytes in the peripheral blood smear may strengthen this impression, but the Paul-Bunnell reaction is consistently negative (Beverley and Beattie, 1958). Rarely, the changes in the peripheral blood may be so striking as to lead to a diagnosis of acute lymphatic leukaemia, as in Case 2 of Kayhoe *et al.* (1957). It should be noted that the E.S.R. is seldom much raised and anaemia is usually absent.

Although the clinical findings may arouse the suspicion of malignant lymphoma, the establishment of this diagnosis is dependent upon the findings in a lymph node biopsy. That there is a possibility of an erroneous diagnosis at this stage is shown by the history of Case 1, where initially a diagnosis of Hodgkin's disease was made on the lymph node biopsy. It is understandable that toxoplasmic lymphadenitis might be mistaken histologically for Hodgkin's disease, lymphosarcoma, or reticulosarcoma, and several authors (Stanton and Pinkerton, 1953; Paton, Dick, and Beverley, 1958; Paterson, 1960) mention that the first impression gained from histological examination of the lymph node biopsy was one of a malignant condition. Pace and Babando (1960) go so far as to state that glandular toxoplasmosis cannot with certainty be distinguished from Hodgkin's disease on the histological findings.

Confusion with malignant lymphomatous conditions is liable to arise from (1) the apparent disorganization of the architecture of the node, (2) the active proliferation of large cells, variously interpreted as lymphoblasts or reticulum cells, (3) the simulation of neoplastic infiltration, and (4) the prominent histiocyte clusters, which resemble those sometimes found in association with Hodgkin's disease and, less often, with lymphosarcoma and reticulosarcoma.

The chief points which distinguish toxoplasmic lymphadenitis from the various forms of malignant lymphoma are:—(1) The lymph node architecture is not, in fact, completely destroyed; (2) the proliferating cells show normal mitoses only and amongst these cells there is a high incidence of necrosis, witnessed by the numerous pyknotic nuclear fragments; (3) giant reticulum cells characteristic of Hodgkin's disease are absent (Bang, 1957); (4) the lymph sinuses are crowded with macrophages; and (5) the cellular infiltration, which involves the capsule and extends outside the node, has all the characteristics of an inflammatory infiltration.

It is too early yet to decide whether the concurrence of toxoplasmosis and lymphosarcoma, or reticulosarcoma, which has been reported twice (see references given by Remington *et al.*, 1960) is more than a chance association. In the case of Finckh (1954), the diagnosis of toxoplasmosis rested solely on the morphological interpretation of organisms seen in bone marrow smears. Certainly this protozoon does not seem to be as frequently associated with the malignant lymphomata as are some fungal infections.

Whilst it is generally agreed that toxoplasmic lymphadenitis can be distinguished histologically from the various forms of malignant lymphoma, there is no unanimity of opinion on the question of whether the histological picture is at all diagnostic of toxoplasmosis. Some authors have stated that the lymph node changes are non-specific or inconstant (Gard and Magnusson, 1951; Ström, 1951; Skipper *et al.*, 1954; Alexander and Callister, 1955; Beverley, Caley, and Warrack, 1958; Paton *et al.*, 1958). According to others, the changes are, at least, highly suggestive of the disease (Wahlgren, 1951; Siim, 1953; Stanton and Pinkerton, 1953; Bang, 1953, 1957; Saxén and Saxén, 1959).

The prominent histiocyte clusters have attracted most attention and these have been described by almost all writers. Some have likened them to 'miniature sarcoid lesions' (Stanton and Pinkerton, 1953), but, as pointed out above, the cell clusters are generally less well defined than sarcoid lesions and there is no increased production of collagen or reticulin (Piringer-Kuchinka, 1953). Thus there is little likelihood of confusion with sarcoidosis.

Robb-Smith (1947) described similar reactive lesions in lymph nodes under the title of lympho-histiocytic medullary reticulosis. He stated that much the same appearance was seen in Leishmaniasis, but in that condition Leishman-Donovan bodies could be identified in the epithelioid histiocytes. Later, Robb-Smith (quoted by Beverley and Beattie, 1958) examined a number of lymph node biopsies from proven cases of toxoplasmosis and reported that, in nine out of 11 cases, the appearances corresponded to his description of lympho-histiocytic medullary reticulosis. He doubted, however, whether the lesion could be regarded as specific for toxoplasmosis.

Similarly, Piringer-Kuchinka (1953) gave an accurate account of the histological changes, including the macrophage infiltration of the lymph sinuses, before she was aware of the occurrence of this type of lesion in toxoplasmosis. In 1958, the same author published an account of 62 cases showing this histological picture. Among these, positive serological tests for toxoplasmosis were obtained in 46 out of the 49 individuals tested (Piringer-Kuchinka *et al.*, 1958).

Bang (1953 and 1957) drew attention to the 'focal medullary reticulosis' which he claimed to be characteristic of toxoplasmic lymphadenitis, and Siim (1953) found the 'same characteristic histological picture' in all of 16 cases where lymph node biopsies were performed. More recently Saxén and Saxén (1959) and Roth and Piekarski (1959) have confirmed the constancy of the histological findings in toxoplasmic lymphadenitis.

Confirmation of the diagnosis has been obtained in some instances by successful isolation of the organism from lymph nodes which have shown

these changes (Lelong, Desmonts, Le Tan Vinh, Nézelof, Satgé, and Couvreur, 1954; Siim, 1956). It must be admitted, however, that, in most of the published series of cases the selection of cases has been made on a histological basis and the diagnosis has been confirmed retrospectively by serological tests. Allowance must therefore be made for the possibility that cases which did not present characteristic histological changes in the lymph node biopsy may have escaped recognition. Nevertheless, this cannot invalidate the conclusion that, when the lymph node biopsy does show this combination of changes, it provides strong presumptive evidence of toxoplasmic lymphadenitis. This conclusion is supported by the findings in the three cases presented here, as well as by the other evidence cited above.

The diagnosis should never rest on the histology alone, for, however characteristic the changes may be, they can hardly be said to be specific. Ideally, confirmation of the diagnosis should be obtained by isolation of the organism, which may be done by intraperitoneal inoculation of the mouse with fresh lymph node suspension (Siim, 1956). Unfortunately, the diagnosis is seldom suspected at the time the lymph node is removed for biopsy and it is hardly justifiable to perform a second operation for this purpose. Moreover, animal inoculations have not proved uniformly successful even when the diagnosis has not been in doubt. Cathie (1954) has successfully isolated toxoplasma by mouse inoculation with the patient's saliva.

In the majority of cases the diagnosis of toxoplasmic lymphadenitis has been confirmed by serological tests, and studies have shown that the results of these are sufficiently reliable for practical purposes (Cathie, 1957). The demonstration of a rising titre in the cytoplasm-modifying antibody (dye test) and the complement-fixation test, or the presence of a high titre in the two tests, is generally taken as evidence of an active infection. These results, taken in conjunction with the characteristic findings in a lymph node biopsy, provide sufficient grounds for a diagnosis of toxoplasmic lymphadenitis.

Only very rarely are toxoplasma cysts to be found in lymph node sections. Indeed, a definite cyst with a clearly defined wall, such as was found in Case 1 of the present series, has been described in a lymph node on only one previous occasion (Stanton and Pinkerton, 1953). In multiple sections of the node, these authors succeeded in demonstrating two such cysts both apparently situated in lymph sinuses. These cysts appear to correspond to the true toxoplasma cyst, described by Lainson (1958), as occurring in chronic toxoplasma infections in animals. They differ from the pseudocysts, which are found in more acute infections, in that there is a clearly defined cyst wall, apparently of parasitic origin. The contained toxoplasms are both smaller and much more numerous than those in the pseudocyts (Lainson, 1958). Most authors have been understandably cautious in identifying vegetative forms of toxoplasma in lymph nodes, in view of their similarity to particles of nuclear débris which are regularly to be found in large numbers.

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Primary carcinoma multiplex

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SYNOPSIS During the past 11 years almost 4,000 necropsies have been performed at the Southern General Hospital, Glasgow, and of these, 924 showed malignancy of which 45 were of primary carcinoma multiplex. Two cases showed triple and one quadruple malignancy, and the remainder two primary malignant tumours.

Primary cancer occurring in more than one organ or in more than one site in a single organ is no rarity. Slaughter (1944) reviewed the literature and collected 1,868 such cases. There are, however, few British references.

The idea of a unicentric origin of carcinoma is gradually giving place to the view that a multicentric origin is not uncommon, and that cancer, so far from being a local disease, is more of the nature of a general condition.

Billroth (1879) was the first to record multiple malignancy. He described two cases and suggested that certain conditions had to be satisfied before a diagnosis of multiple primary tumours could be established. Not only must tumours arise in different situations but they should have an independent histological appearance and each tumour should be capable of producing its own metastases.

It is thought that similar growths occurring in the same tissue or in bilateral organs, such as the breast and kidney, should be debarred and counted as one. The skin should also be regarded as a single organ except where the tumours are of obviously different histogenesis.

MATERIAL

Between January 1950 and December 1960 3,771 necropsies were performed at the Southern General Hospital, Glasgow. In 924 patients malignant disease was found (Table I). Of these, 879 showed one primary neoplasm, 42 showed two, two showed three, and one showed four (Table II), an incidence of 4·87% with multiple carcinoma. The site distribution of tumours in the 42 patients with two primary malignant tumours is shown in Fig. 1. The sex distribution, although differing slightly from

TABLE I

SEX DISTRIBUTION

Sex	*Necropsies Showing Cancer*	*No. of Primary Tumours*
Male	533	555
Female	391	418
Total	924	973

TABLE II

INCIDENCE OF MULTIPLE PRIMARY TUMOURS

No. of Primary Tumours	*Male*	*Female*	*Total*
Single	513	366	879
Double	18	24	42
Triple	2	—	2
Quadruple	—	1	1
Total	533	391	924

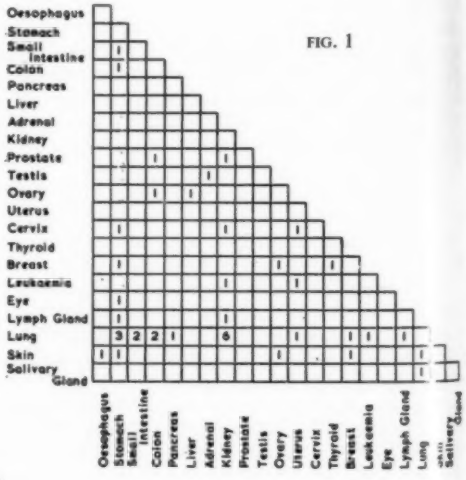

FIG. 1

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the previous American series, shows, as did Pennell (1958), a slight predominance in females (24 females as to 18 males).

The case reports of the three cases of primary carcinoma multiplex will be given more fully.

CASE REPORTS

CASE 1 A 78-year-old retired cobbler was admitted to hospital (October 1959) in very poor general condition, complaining of general weakness, anorexia, and diarrhoea. He was found to have obvious weight loss and pressure sores over the sacrum. Abdominal examination revealed a palpable epigastric mass suggesting a tumour of the alimentary tract. He was not fit for detailed investigation, and steady deterioration continued till his death two weeks later.

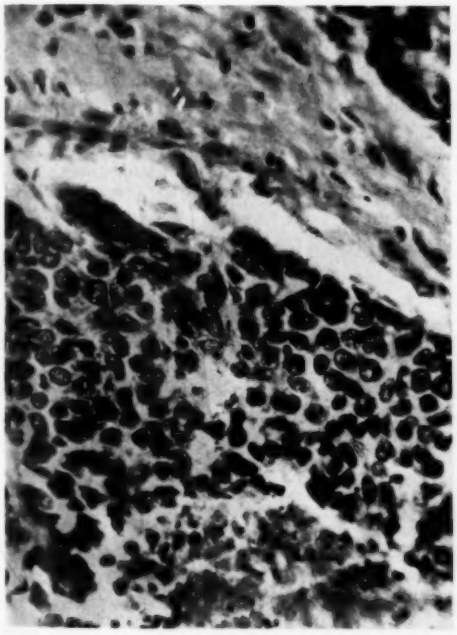

FIG. 2. *Histological examination of the oesophageal tumour in Case 1 confirms the presence of a poorly differentiated squamous cell carcinoma. Haematoxylin and eosin × 480.*

Relevant necropsy findings The oesophagus showed, 4 in. from the cardia, a fungating neoplasm stretching from that point to within 1 in. of the cardia. Histologically, this was a poorly differentiated squamous cell carcinoma (Fig. 2).

The upper abdominal mass was found to be a carcinoma of the head of the pancreas (Fig. 3) and histological examination revealed an adenocarcinoma of

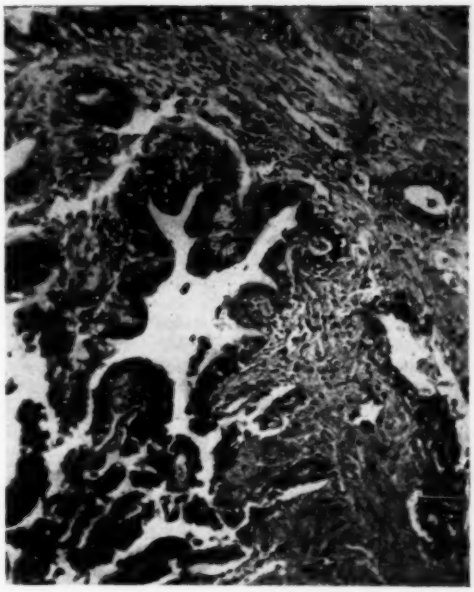

FIG. 3. *The pancreas of Case 1 shows extensive infiltration with adenocarcinoma of ductal origin. Haematoxylin and eosin × 140.*

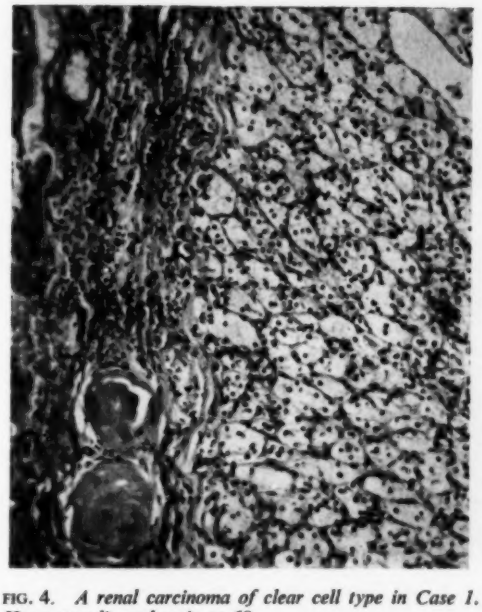

FIG. 4. *A renal carcinoma of clear cell type in Case 1. Haematoxylin and eosin × 68.*

ductal origin. On the cortical surface of the right kidney there appeared to be a small adrenal rest, but histologically (Fig. 4) this was found to be a clear cell renal carcinoma.

CASE 2 A 77-year-old, healthy looking man was admitted to hospital in July 1958 on account of increased frequency of micturition associated with painless haematuria of seven months' duration. The bladder was not distended and there were no abnormal palpable masses. Rectal examination disclosed a smooth firm enlargement of the prostate with one hard nodule. The blood urea was 65 mg. %. An intravenous pyelogram showed no radio-opaque renal calculi and that both kidneys were excreting satisfactorily. The urinary bladder did not fill completely and was thought to be due to extrinsic causes. Cystoscopy, however, revealed widespread papillomata of the bladder, biopsy of which showed malignant change. The patient collapsed and died suddenly 12 days later.

Necropsy At necropsy the cause of death was found to be multiple small bilateral pulmonary emboli. At the upper pole of the left kidney there was a firm white tumour 3 cm. in diameter. Histologically this was a clear cell renal carcinoma. Microscopic examination of the prostate revealed primary adenocarcinoma and of the bladder papillary carcinoma of transitional cell type.

CASE 3 In October 1957 a 65-year-old woman had a right hemicolectomy performed for a primary carcinoma of caecum (Fig. 5). In December 1957 a left radical mastectomy was carried out for a primary carcinoma of breast; the mastectomy was followed by radiotherapy. In February 1960, a rodent ulcer at the inner canthus of the left eye was treated by radiotherapy. In March 1960 she returned with symptoms of intestinal obstruction. Laparotomy was performed shortly after her admission. The lower colon was the site of an extensive tumour causing the obstruction and a transverse colostomy was performed. Suddenly on the eighteenth post-operative day she developed acute chest pain, collapsed, and died in a few moments.

Necropsy At necropsy, the primary cause of death was pulmonary embolism. There was a soundly healed left mastectomy scar and post-irradiation scarring of the left inner canthus at the site of the previously treated rodent ulcer. A simple colloid adenoma was found in the right lobe of the thyroid. The pelvic tumour mass was a large, fungating, well-differentiated primary adenocarcinoma of the rectum (Fig. 6). Secondary adenocarcinoma was found in the pelvic lymph glands but there was no other intra-abdominal secondary tumour. Apart from six simple jejunal diverticulae, there was no other abnormality of the alimentary tract. The uterus, on section, showed two simple fundal leiomyomata. On section of the brain, a small circular tumour, 1·5 cm. in diameter, was situated in the posterior commissure. Histologically this nodule showed secondary infiltration

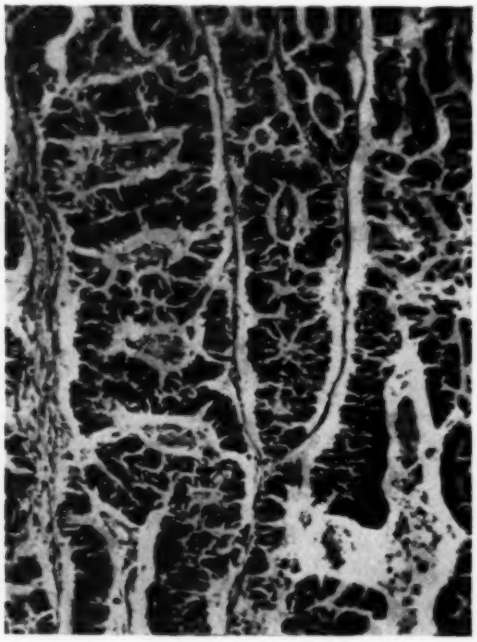

FIG. 5. *An adenocarcinoma of caecum in Case 3. Haematoxylin and eosin × 140.*

FIG. 6. *A well-differentiated adenocarcinoma of the rectum in Case 3. Haematoxylin and eosin × 140.*

TABLE IV

COMPARISON OF FIGURES FOR CARCINOMA MULTIPLEX FROM VARIOUS CENTRES

Centre	*Date*	*Total Malignant Tumours*	*Multiple Primary Malignant Tumours*	*Author*
Leningrad	1931	1,238	23 (1·8%)	Goriainowa and Schabad
Harvard University	1932	1,078	40 (3·7%)	Warren and Gates
Mayo Clinic	1933	2,124	71 (3·3%)	Hurt and Broders
Michigan University	1934	983	30 (3·1%)	Bugher
Wisconsin University	1936	583	46 (7·8%)	Burke
London	1957	3,096	299 (9·7%)	Wallace
Glasgow	1961	973	94 (9·6%)	Present series

with a spheroidal cell carcinoma suggesting an origin from breast.

There was quite clear evidence in the section that there was a cribriform intraduct carcinoma as well as multiple papillomata throughout the ducts. The mammary tumour showing these duct changes demonstrated their local origin.

DISCUSSION

Pathologists have been interested for many years in the study of multiple malignant primary neoplasms (Table IV). This subject is of considerable importance to surgeons and was discussed by them in London in 1930 (Norbury) and again in 1944 (Whigham). These, together with the publications of Williamson (1950), Wallace (1957), and Pennell (1958), appear to be the only reviews in the British literature. Innumerable isolated cases have, however, been described in all parts of the world during this and the last century. Our own results are compared in Table IV with those from other centres, the Glasgow figure of 9·6% being paralleled by the London percentage.

Thomas, Dockerty, and Waugh (1948) concluded that multiple primary carcinoma is a reflection of an inherent constitutional susceptibility to cancer which is present in some individuals. Ewing (1928) suggested that one malignant growth in an individual provides fertile soil for the development of a second tumour. This implies that the risk of developing cancer is not evenly spread over the population, and that some individuals are more susceptible than others or are more exposed to influences bringing about its development.

Mider, Schilling, Donovan, and Rendall (1952) suggested that the probability that an individual with one primary neoplasm is more likely to develop a second one, than could be accounted for by mere chance, is favoured by Warren and Gates (1932), Mider *et al.* (1952), and Barrett, Miller, and Fessenmeyer (1949). Watson (1953), on the other hand, came to the conclusion that there was no evidence of a tendency to develop a second growth, nor was there any evidence that one growth conferred a relative immunity against plurality.

The occurrence of multiple cancers is becoming an increasingly important clinical fact because an ageing population has a higher incidence of carcinoma. Already facilities for early diagnosis, modern radiotherapy and chemotherapy, and safer anaesthesia have encouraged a more radical approach to treatment, and with initial tumours thus ablated, or held in check, even more cases of multiple malignancy may occur.

We are indebted to Dr. A. Dick and Mr. R. B. Wright for their criticism and advice during the preparation of this paper, and to Mr. G. F. Headden for technical and photographic assistance.

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A simple method of weighing the heart

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SYNOPSIS A simple method of dividing and weighing the heart is described. It is quick to perform and ratios between the left and right ventricles and the total weight of the heart are remarkably constant.

Analysis of 788 cases shows that these ratios are of value in the diagnosis after death of systemic and pulmonary hypertension. This is applicable to both sexes at all ages.

An unexpected finding was a significant correlation between pulmonary hypertension and acute massive pulmonary embolism, of which the prophylactic and therapeutic implications are mentioned.

The value of the method in childhood and infancy is indicated.

Weighing the heart is a usual procedure in routine necropsies but its value is restricted in any particular case by ignorance of the normal weight and by the difficulty of determining the contribution of each part of the heart to a variation from the supposed normal. A method of dividing the organ in order to weigh its various parts was described by Lewis (1914) but the procedure takes some weeks to complete and this limits its value in routine work. Some pathologists estimate hypertrophy of the ventricles by measuring the thickness of their walls but in our hands this has not been successful owing to the irregularity of the endocardial surfaces. Others assess the presence of pulmonary hypertension by measurements of the pulmonary vessels in histological sections (Heath and Whitaker, 1955) but we have found this unsatisfactory for routine work.

Some years ago a standard technique for dividing and weighing the heart was adopted, and having collected data from 788 cases the findings are presented here in the hope that the method will be useful to others. The technique was purposely made as simple as possible as time is often limited in routine work and great accuracy was deliberately sacrificed for the same reason. The whole procedure takes only a few minutes and results in fairly comparable figures. The whole of the interventricular septum is weighed with the left ventricle and no attempt is made to separate the two auricles; the pericardial fat and coronary vessels are not removed from the surface of the heart. It was intended to avoid defining the weight of the normal heart by calculating the ratios of the weights of its various parts.

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METHOD

Although Lewis (1914) explains his method at length, Herrmann and Wilson (1922) found that his directions were not explicit enough to prevent misunderstanding. Our technique is therefore set out below in full detail of each step we actually take and illustrated in Figs. 1 to 3.

1 The thoracic contents are removed *in toto* and the heart is not removed from them until step 7 below. Running water is used when required throughout the examination.

2 After examining the remainder of these organs and opening the pericardium to its apex, the right auricle is opened with scissors and examined with the tricuspid valve.

3 A long knife is then passed through the tricuspid orifice to the apex of the right ventricle and this is opened by an incision along the length of its extreme outer border.

4 With scissors laid flat against the right ventricular surface of the interventricular septum, the right ventricle is cut off it from apex to base along its anterior junction with it. None of the right ventricle now remains attached anteriorly to the septum, and the incision is continued through the pulmonary valve and into the pulmonary artery and its branches (Fig. 1).

5 Having examined the right chambers, steps 2 and 3 are now copied exactly on the left side thus exposing the left auricle, mitral valve, and most of the left ventricle.

6 The aortic valve and thoracic aorta are now opened by cutting upwards through the mitral valve and the aortic valve with scissors.

7 The heart is now removed from the other viscera by lifting it vertically, apex upwards, with the remainder

FIG. 1. *Cutting the right ventricle from the right side of the septum anteriorly.*

FIG. 2. *Removing the heart from the other thoracic viscera. Note the manner in which the anterior edge of the right ventricle has been cut from its junction with the septum.*

of the thoracic viscera hanging from it. The pulmonary veins and venae cavae are now divided at their junctions with the auricles, and the aorta and pulmonary arteries are divided at the level of the upper edges of their valve cusps. It is most important that these vessels be divided at these respective levels and we draw attention to the possibility of error at this stage (Fig. 2).

8 All the remaining blood clot is washed away and lightly adherent thrombus removed with forceps. The heart is now weighed.

9 The coronary arteries are now opened and examined as may be desired.

10 The posterior attachment of the right ventricle to the septum is now divided, again with scissors laid flat against the right ventricular surface of the septum.

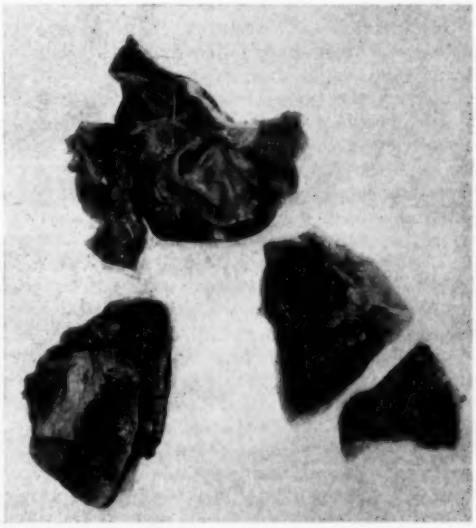


FIG. 3. *The heart as finally divided. Above, the auricles; below on the left, the left ventricle; below on the right, the right ventricle in its two parts.*

11 The auricles are now removed by cutting them off the ventricles or pieces of ventricle along the coronary sulcus, and joining these incisions across the top of the septum. The heart is thus divided into four pieces (*a*) the auricles, (*b*) the left ventricle attached to the septum, and (*c*) the right ventricle in two pieces. The two ventricles are weighed, recording (*b*) as the left ventricle and (*c*) as the right (Fig. 3). Simple ratios are calculated as follows to one place of decimals.

$$\frac{\text{Weight of left ventricle}}{\text{Weight of right ventricle}} = \text{L/R}$$

$$\frac{\text{Weight of whole heart}}{\text{Weight of right ventricle}} = \text{T/R}$$

$$\frac{\text{Weight of whole heart}}{\text{Weight of left ventricle}} = \text{T/L}$$

The weight of the auricles can be determined by subtraction.

RESULTS

The material available consists of the data from 788 post-mortem examinations, being all the routine necropsies performed at one hospital in the years 1954-57, except for a small number which had to be discarded on the grounds of insufficient information. There are 650 adults (429 of them males), 20 adolescents aged 10 to 20 years, 23 children aged 1 to 10 years, and 95 infants under 1 year of age. Most of the discards were infants whose hearts had not been divided.

Our attention was first directed to adults and the findings were considered for each sex in age groups. As usual in routine hospital work there is a preponderance of cases in the sixth and seventh decades. The numbers in the younger age groups from which it might be expected to discover the normal values are small but from them and from accident cases ideas were formed as to the probable directions in which deviations might be expected to occur. It became necessary to place an arbitrary maximum for the weight of the normal heart in each sex in order to determine the approximate normal values for the new measurements. There is no real agreement on the maximum weight of the human heart (Zeek, 1942) and it has been the custom for many years in our department to regard it as 350 g. for males and 300 g. for females. As we have no facilities for weighing the bodies (Bartels and Smith, 1932), these figures were taken as normal and have proved satisfactory. Indeed, raising the values to 400 g. and 350 g. respectively did not greatly affect the overall results but did include a number of clinically abnormal hearts in the normal groups.

DISTRIBUTION OF HEART WEIGHTS At this point it seems worth while to examine the distribution of heart weights in this material. Largely on the basis of the clinical history in each case and with due regard to the general post-mortem findings, all the adult hearts (20 years and above) were divided into three populations.

1 *No vascular disease or normal* This refers in fact to the absence of systemic hypertension or chronic pulmonary disease (see 3 below), and includes conditions other than hypertension which might give rise to cardiac hypertrophy, *e.g.*, aortic valvular stenosis, pericarditis, etc.

2 *Systemic hypertension* A clinical history consistent with it and blood pressure above 140/90 mm. Hg.

3 *Pulmonary disease excluding hypostatic pneumonia or single attack of lobar pneumonia* Conditions which might reasonably be expected to give rise to pulmonary hypertension such as mitral stenosis were included. When systemic hypertension was also present, the case was placed in group 2.

Figure 4 shows the distribution of total heart weights in these three classes. There is some tendency to support our arbitrary estimate of normal heart weight, since in males the hearts of only eight (3·5%) of cases of systemic hypertension fall below 350 g. and in females only five (5·5%) fall below 300 g. It is possible that in some of these patients a sufficient degree of cardiac hypertrophy had not been achieved to bring the heart weight above these values. It is admitted that in each sex roughly one third of the 'normals' is found above these weights but these, of course, include hearts enlarged for some reason other than hypertension and those where there was insufficient evidence for a firm diagnosis of hypertensive disease. In both sexes the cases of pulmonary disease are fairly evenly distributed throughout the range; total heart weight is little help in the diagnosis of pulmonary hypertension.

In normal hearts the weight of the right ventricle by our method is about 80 to 100 g. and the left ventricle weighs about 100 g. more. In females these figures are smaller by some 20 g. The weight of the auricles was more variable and we have failed to attach any significance to these variations. In normal hearts of whatever weight and in both sexes the ratios were remarkably constant as follows:—L/R 2 to 3, T/R 3 to 5, and T/L 1·5 to 2.

In left ventricular hypertrophy it would be expected that the first two ratios would become larger and the last smaller. In known cases of systemic hypertension or aortic valvular disease L/R and T/R were found to be increased beyond 3 and 5 respectively, though in some cases only one was raised. It seemed therefore that when L/R equals or exceeds 3 and/or T/R equals or exceeds 5 (values 'raised' or 'increased') there is left ventricular hypertrophy associated with systemic hypertension unless some other explanation can be found.

Lewis (1914) remarks that the commonest type of cardiac hypertrophy is a uniform hypertrophy and in this case these ratios could be either normal or increased.

In the conditions which are usually associated with pulmonary hypertension such as mitral stenosis, Fallot's tetralogy, and pulmonary fibrosis the ratio T/L is greater than L/R or equal to it ('ratios reversed') and this was the case in 58% of infants mostly in the first few days of life (Table IV). Such a deviation would be expected in right ventricular

FIG. 4.—Number of cases found at each Heart Weight. [Male adults.]

PULMONARY DISEASE
SYSTEMIC HYPERTENSION
NORMALS
NUMBER OF CASES
TOTAL WEIGHT OF HEART (G.)
200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 & OVER

FIG. 4a.—*Number of cases found at each Heart Weight. [Male adults.]*

FIG. 4b.—*Number of cases found at each Heart Weight. [Female adults]*

hypertrophy and it was therefore concluded that it indicates pulmonary hypertension if other explanations are lacking.

In order to test the validity of these two conclusions the adult cases were divided first into two main categories, those with normal and those with increased heart weights using the arbitrary standards. Each category was subdivided into three groups, namely, (*a*) ratios normal L/R<3, T/R<5, and T/L<L/R; (*b*) ratios raised L/R≥3, and/or T/R≥5, T/L<L/R; (*c*) ratios reversed T/L≥L/R (D≤0).

The 788 cases were allocated to these groups in the three categories as defined above, those having no vascular disease, those having clinical systemic hypertension, and those dying of pulmonary disease excluding terminal or hypostatic pneumonia. The few cases of mitral stenosis or Fallot's tetralogy were included as pulmonary disease. Being puzzled as to the place of acute pulmonary embolism, such cases were at first placed in a separate category. The tabulated results for the two sexes in three age groups are shown in Table I. The results of simple significance tests on these figures are also shown and they tend to confirm our conclusions that 'raised' values of L/R and/or T/R are associated with systemic hypertension while 'ratios reversed' in which D (L/R-T/L) is 0 or less than 0 are associated with pulmonary hypertension. The ratios L/R, T/R, and for D (L/R-T/L) were therefore analysed further as shown in Figs. 5, 6, and 7. It can be seen that (1) L/R is 3 or more in only 25 (15%) of males and in 15 (14%) of females with no hypertensive

TABLE I

CLASSIFIED RESULTS IN ADULTS AND NUMBER OF CASES IN EACH CATEGORY

		20 to 49 Years			*50 to 69 Years*			*70 Years and Over*			*Grand Total*	*Grand Total allocation to Carcinoma*
		Male	*Female*	*Total*	*Male*	*Female*	*Total*	*Male*	*Female*	*Total*		
T. normal Ratios normal	No vascular disease[1]	29 (88)	24 (100)	53 (94)	49 (74·5)	15 (68·1)	64 (73)	17 (77·5)	5 (62·5)	22 (73·2)	139 (79·5)	155 (88·5)
	Systemic hypertension[2]	0	0	0	3 (4·5)	0	3 (3·4)	1 (4·5)	1 (12·5)	2 (6·7)	5 (2·9)	6 (3·5)
	Pulmonary disease[3]	3 (9)	0	3 (5·2)	13 (19·5)	5 (22·8)	18 (20·2)	4 (18)	0	4 (13·4)	25 (14·2)	8 } 14 (8
	Pulmonary embolism	1 (3)	0	1 (1·8)	1 (1·5)	2 (9·1)	3 (3·4)	0	2 (25)	2 (6·7)	6 (3·4)	6 } (3
	Total	33	24	57	66	22	88	22	8	30	175	175
T. normal Ratios raised	No vascular disease	1 (50)	5 (71·4)	6 (66·7)	10 (62·5)	5 (50)	15 (57·7)	5 (50)	3 (60)	8 (53·4)	29 (58)	29 (58)
	Systemic hypertension	1 (50)	2 (28·6)	3 (33·3)	4 (25)	5 (50)	9 (34·6)	4 (40)	2 (40)	6 (40)	18 (36)	19 (38)
	Pulmonary disease	0	0	0	2 (12·5)	0	2 (7·7)	1 (10)	0	1 (6·6)	3 (6)	2 } 2 (4
	Pulmonary embolism	0	0	0	0	0	0	0	0	0	0	0 }
	Total	2	7	9	16	10	26	10	5	15	50	50
T. normal Ratios reversed	No vascular disease	0	0	0	1 (8·3)	2 (22·2)	3 (14·2)	1 (16·7)	3 (100)	4 (44·5)	7 (20)	12 (34·4)
	Systemic hypertension	0	0	0	0	0	0	0	0	0	0	1 (2·8)
	Pulmonary disease	3 (100)	2 (100)	5 (100)	10 (83·4)	2 (22·2)	12 (57·4)	4 (66·6)	0	4 (44·5)	21 (60)	15 } 22 (42
	Pulmonary embolism	0	0	0	1 (8·3)	5 (55·6)	6 (28·4)	1 (16·7)	0	1 (11)	7 (20)	7 } (20
	Total	3	2	5	12	9	21	6	3	9	35	35
T. raised Ratios normal	No vascular disease	5 (18·5)	5 (50)	10 (27·1)	17 (26·2)	4 (12·5)	21 (21·6)	4 (16·6)	3 (15)	7 (15·9)	38 (21·4)	41 (23)
	Systemic hypertension	22 (81·5)	3 (30)	25 (67·6)	32 (49·2)	20 (62·5)	52 (53·6)	16 (66·8)	14 (70)	30 (68·2)	107 (60·1)	108 (60·7)
	Pulmonary disease	0	1 (10)	1 (2·7)	10 (15·4)	2 (6·3)	12 (12·4)	2 (8·3)	0	2 (4·5)	15 (8·4)	11 } 29 (6
	Pulmonary embolism	0	1 (10)	1 (2·7)	6 (9·2)	6 (18·7)	12 (12·4)	2 (8·3)	3 (15)	5 (11·4)	18 (10·1)	18 } (10
	Total	27	10	37	65	32	97	24	20	44	178	178
T. raised Ratios raised	No vascular disease	2 (14·3)	0	2 (9·4)	2 (5·2)	1 (5·5)	3 (5·3)	8 (25·8)	0	8 (16·4)	13 (10·4)	13 (10·2)
	Systemic hypertension	12 (85·7)	5 (71·4)	17 (81·2)	33 (84·5)	15 (83·5)	48 (84·2)	20 (64·6)	16 (88·9)	36 (73·4)	101 (79·4)	102 (80·4)
	Pulmonary disease	0	2 (28·6)	2 (9·4)	3 (7·7)	1 (5·5)	4 (7·0)	2 (6·4)	0	2 (4·1)	8 (6·3)	7 } 12 (5
	Pulmonary embolism	0	0	0	1 (2·6)	1 (5·5)	2 (3·5)	1 (3·2)	2 (11·1)	3 (6·1)	5 (3·9)	5 } (3
	Total	14	7	21	39	18	57	31	18	49	127	127
T. raised Ratios reversed	No vascular disease	3 (25)	2 (20)	5 (22·7)	7 (20)	3 (37·5)	10 (23·2)	4 (33·3)	3 (37·5)	7 (35)	21 (25·8)	24 (28·2)
	Systemic hypertension	0	0	0	0	0	0	1 (8·4)	0	1 (5)	1 (1·2)	1 (1·2)
	Pulmonary disease	9 (75)	7 (70)	16 (72·8)	26 (74·3)	3 (37·5)	29 (67·5)	5 (41·6)	2 (25)	7 (35)	52 (61·2)	50 } 60 (58
	Pulmonary embolism	0	1 (10)	1 (4·5)	2 (5·7)	2 (25)	4 (9·3)	2 (16·7)	3 (37·5)	5 (25)	10 (11·8)	10 } (11
	Total	12	10	22	35	8	43	12	8	20	85	85

Figures in brackets denotes percentages

Association between increased heart weight and raised ratios L/R and T/R or reversed ratio D (L/R − T/L)

[1]No vascular disease, both sexes $\chi^2 = 0{\cdot}145$ $0{\cdot}8 > P > 0{\cdot}7$ N.S.

[2]Systemic hypertension, both sexes $\chi^2 = 4{\cdot}72$ $0{\cdot}05 > P > 0{\cdot}02$ S.

[3]Pulmonary diseases (pulmonary embolism included), both sexes $\chi^2 = 0{\cdot}001$ $0{\cdot}95 > P > 0{\cdot}90$ doubtful significance

disease (normals) and there are no cases of pulmonary disease in either sex with L/R ⩾3. The cases of systemic hypertension are, however, rather widely scattered. (2) T/R is 5 or more in 44 (22%) of males and in 24 (25%) of females regarded as 'normal'; there is only one case of pulmonary disease, a female. Again systemic hypertension is quite widely distributed. (3) D (L/R-T/L), in which a value of 0 or less includes 48 (87·5%) cases of pulmonary disease in males and 19 (90%) cases in females. In systemic hypertension (which takes precedence when both conditions are present) 5% and 2% respectively fall in this range and in normals the incidence for the two sexes is 1% and 12%.

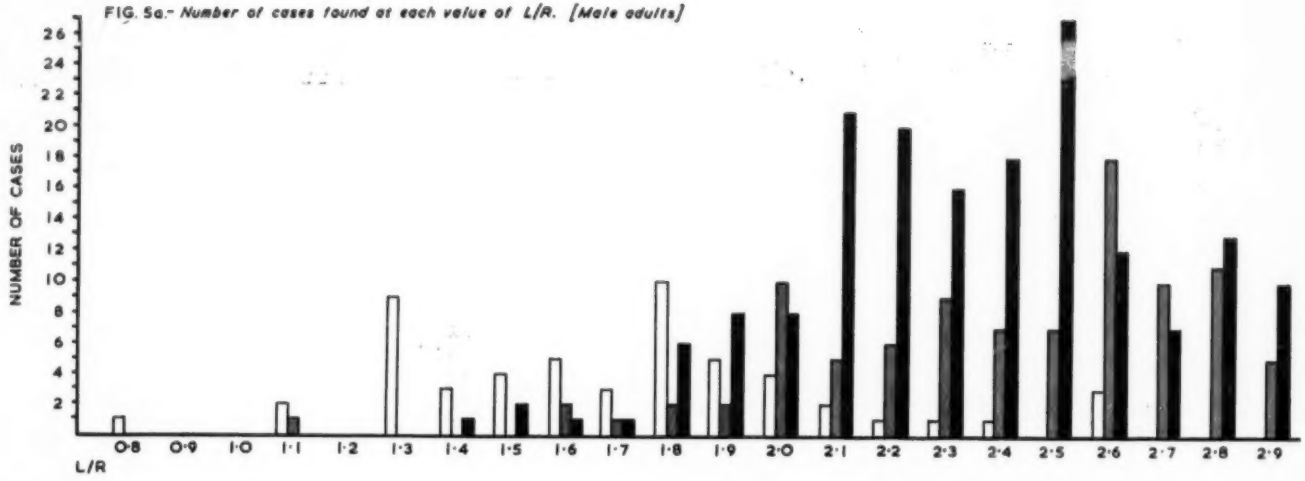
In order to examine further the variability of these three series and of the total heart weights probability curves were constructed using a log. normal scale except in the case of D where there are negative values (Figs. 8 to 11). In each case the 95% range (2·5% to 97·5%) is shown and the median can be derived from them for each category. These curves indicate clearly the variability of each series and very approximate significance tests are shown in Table II. The only occasions when there is no apparent significant variation concern the curves for 'all cases' in T/R and D. It seems reasonable to suppose therefore that the variations in the heart weights in L/R, T/R, and D in the various categories are distinct

TABLE II

ANALYSIS OF FIGURES 8 TO 11

	No. of Cases	*Median*	*95% Range*	*σ (Approximately)*	*Difference of Median from Median of Normal*	*S.E. of Difference*
Heart Weight						
Males						
Normal	195	325	216–495=244	61	—	—
Systemic hypertension	179	430	315–655=340	85	105	± 7·7
Pulmonary diseases	55	425	255–565=310	78	100	±11·4
All cases	429	435	235–600=365	91	110	± 6·2
Females						
Normal	107	274	195–495=300	75	—	—
Systemic hypertension	93	375	255–570=315	79	101	±10·9
Pulmonary diseases	21	345	205–545=340	85	71	±19·9
All cases	221	325	150–600=450	113	51	±10·0
L/R						
Males						
Normal	195	2·37	1·63–3·4=1·77	0·44	—	—
Systemic hypertension	179	2·8	1·63–4·7=3·07	0·77	0·43	±0·07
Pulmonary diseases	55	1·65	1·0–2·33=1·33	0·33	0·72	±0·06
All cases	429	2·45	1·38–4·0=2·62	0·65	0·08	±0·03
Females						
Normal	107	2·35	1·6 –3·55=1·95	0·49	—	—
Systemic hypertension	93	2·85	1·73–4·95=3·22	0·81	0·5	±0·1
Pulmonary diseases	21	1·78	1·3 –2·5 =1·2	0·3	0·57	±0·08
All cases	221	2·35	1·48–4·4 =2·92	0·73	0	—
T/R						
Males						
Normal	195	4·38	3·08–6·05=2·97	0·74	—	—
Systemic hypertension	179	4·9	3·3 –7·5 =4·2	1·05	0·52	±0·095
Pulmonary diseases	55	3·4	2·53–4·45=1·92	0·48	0·98	±0·084
All cases	429	4·5	2·9 –6·8 =3·9	0·98	0·12	±0·071 NS
Females						
Normal	107	4·4	3·25–5·75=2·5	0·63	—	—
Systemic hypertension	93	4·9	3·4 –8·0 =4·6	1·15	0·5	±0·13
Pulmonary diseases	21	3·6	2·4 –5·1 =2·7	0·68	0·8	±0·16
All cases	221	4·4	3·0 –6·3 =3·3	0·83	0	—
D						
Males						
Normal	195	+0·7	−0·4 – +1·75=2·15	0·54	—	—
Systemic hypertension	179	+1·1	−0·5 – +2·7 =3·2	0·8	0·4	±0·07
Pulmonary diseases	55	−0·4	−1·45 – +0·6 =2·05	0·51	1·1	±0·08
All cases	429	+0·7	−0·85 – +2·25=3·1	0·78	0	—
Females						
Normal	107	+0·5	−0·85 – +1·95=2·8	0·7	—	—
Systemic hypertension	93	+1·0	−0·3 – +3·45=3·75	0·94	0·5	±0·12
Pulmonary diseases	21	−0·25	−1·35 – +0·3 =1·65	0·41	0·75	±0·11
All cases	221	+0·6	−0·7 – +2·65=3·35	0·84	0·1	±0·09 NS

FIG. 5a.- *Number of cases found at each value of L/R. [Male adults]*



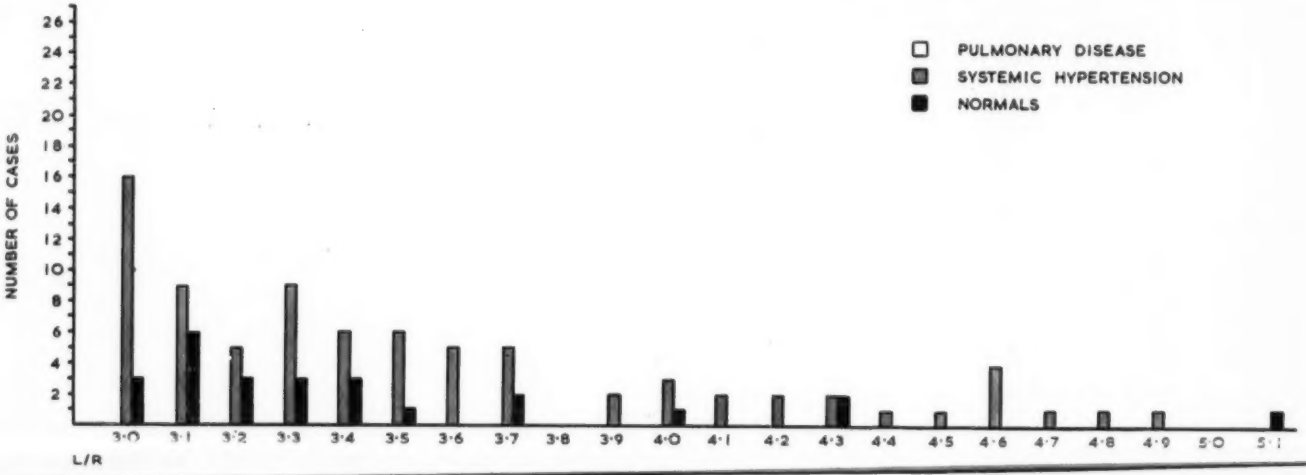

NUMBER OF CASES
L/R
3·0 3·1 3·2 3·3 3·4 3·5 3·6 3·7 3·8 3·9 4·0 4·1 4·2 4·3 4·4 4·5 4·6 4·7 4·8 4·9 5·0 5·1
PULMONARY DISEASE
SYSTEMIC HYPERTENSION
NORMALS

FIG. 5b.-*Number of cases found at each value of L/R. [Female adults]*

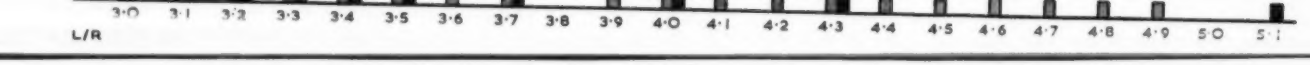
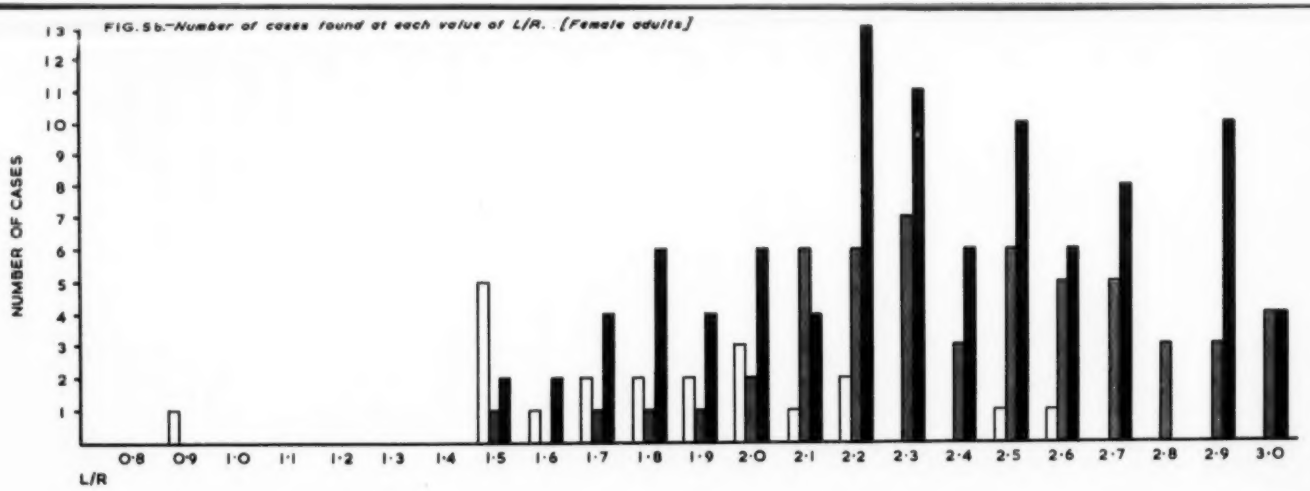


FIG. 5b.—*Number of cases found at each value of L/R. [Female adults]*



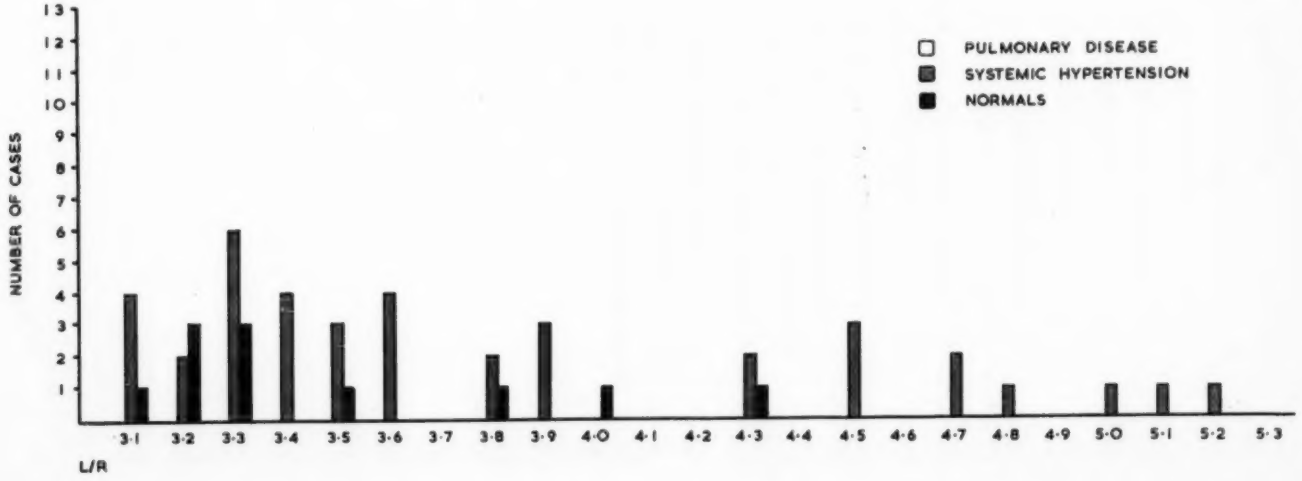
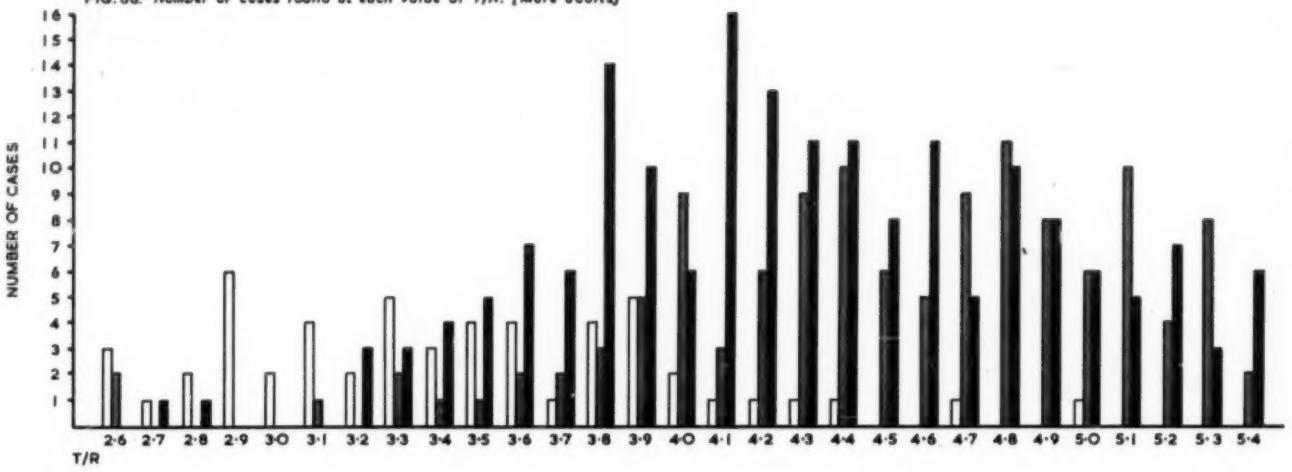


FIG. 6a.—Number of cases found at each value of T/R. [Male adults]



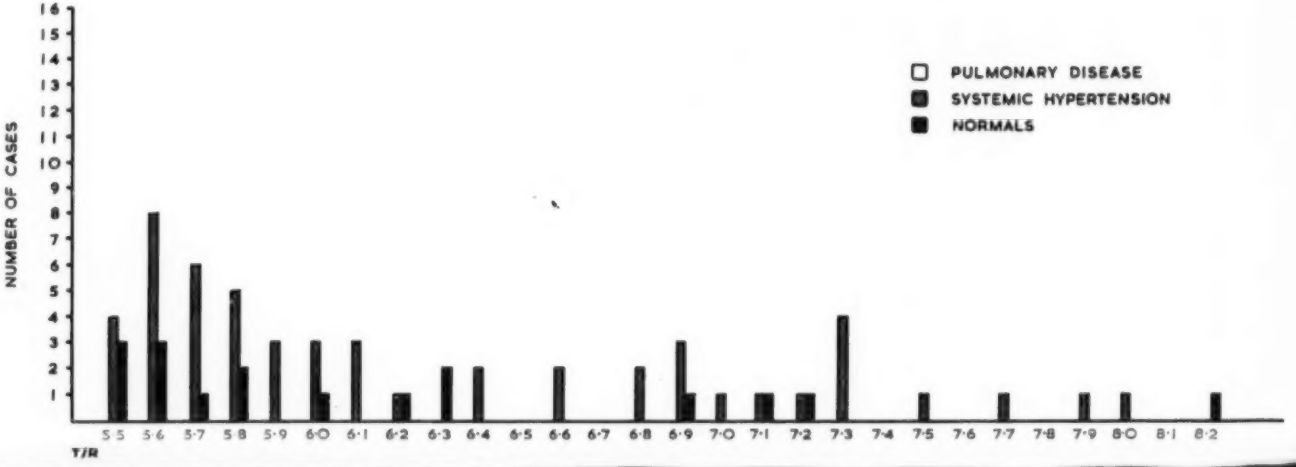
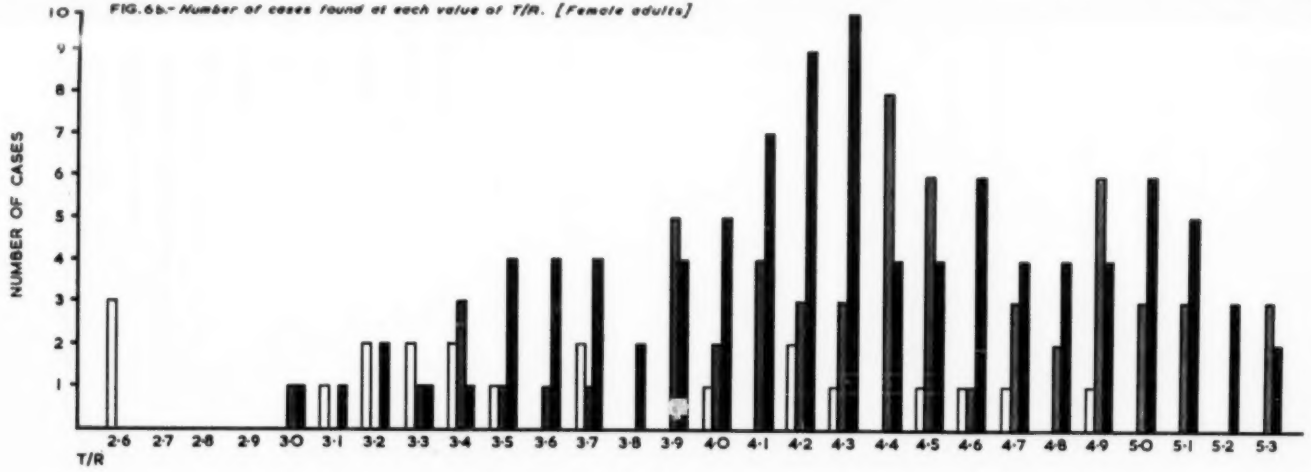
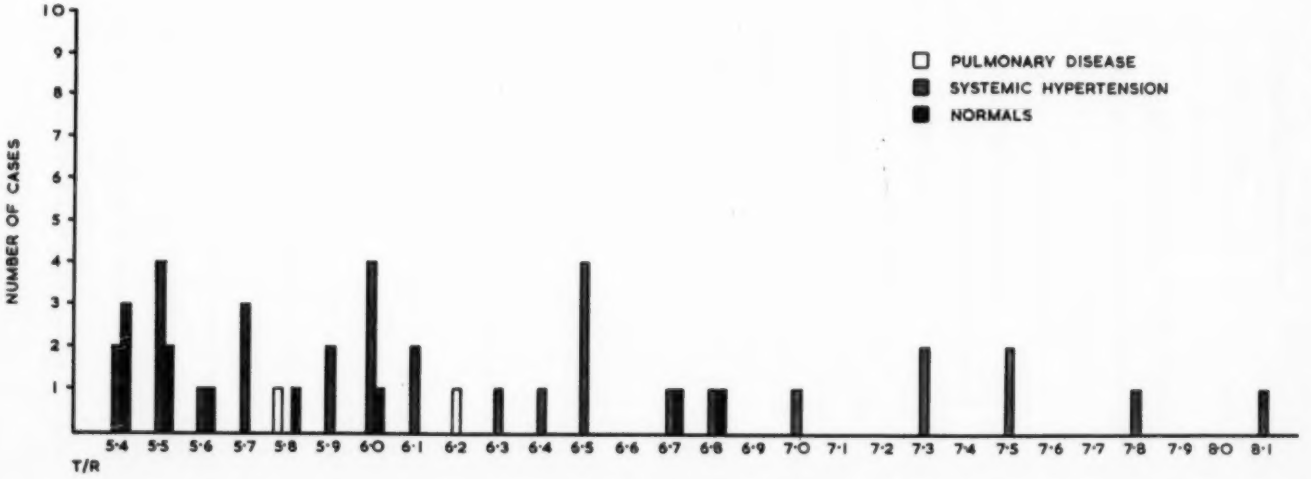

NUMBER OF CASES
T/R
5·5 5·6 5·7 5·8 5·9 6·0 6·1 6·2 6·3 6·4 6·5 6·6 6·7 6·8 6·9 7·0 7·1 7·2 7·3 7·4 7·5 7·6 7·7 7·8 7·9 8·0 8·1 8·2
PULMONARY DISEASE
SYSTEMIC HYPERTENSION
NORMALS

FIG. 6b.—Number of cases found at each value of T/R. [Female adults]

FIG. 6b.- Number of cases found at each value of T/R. [Female adults]




PULMONARY DISEASE
SYSTEMIC HYPERTENSION
NORMALS
NUMBER OF CASES
T/R
5·4 5·5 5·6 5·7 5·8 5·9 6·0 6·1 6·2 6·3 6·4 6·5 6·6 6·7 6·8 6·9 7·0 7·1 7·2 7·3 7·4 7·5 7·6 7·7 7·8 7·9 8·0 8·1

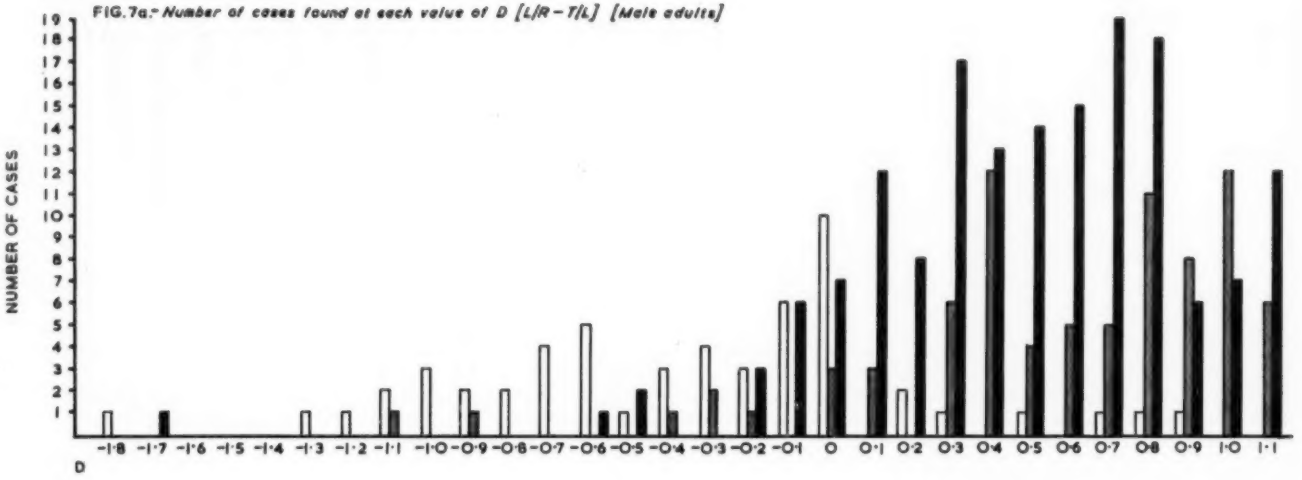


FIG. 7a.—Number of cases found at each value of D [L/R − T/L] [Male adults]

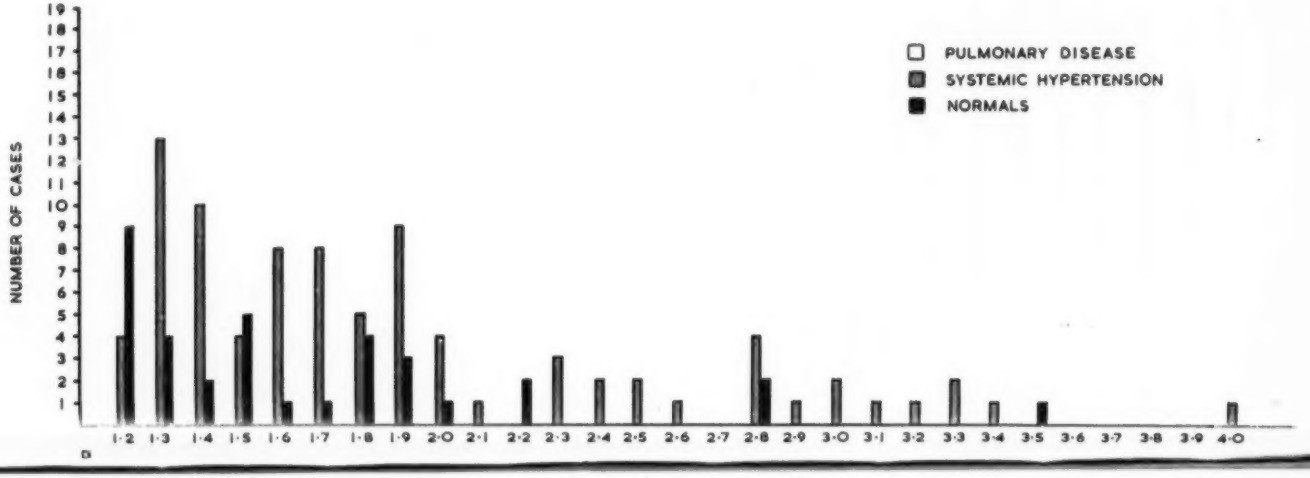


FIG. 7b.—Number of cases found at each value of D [L/R − T/L] [Female adults]

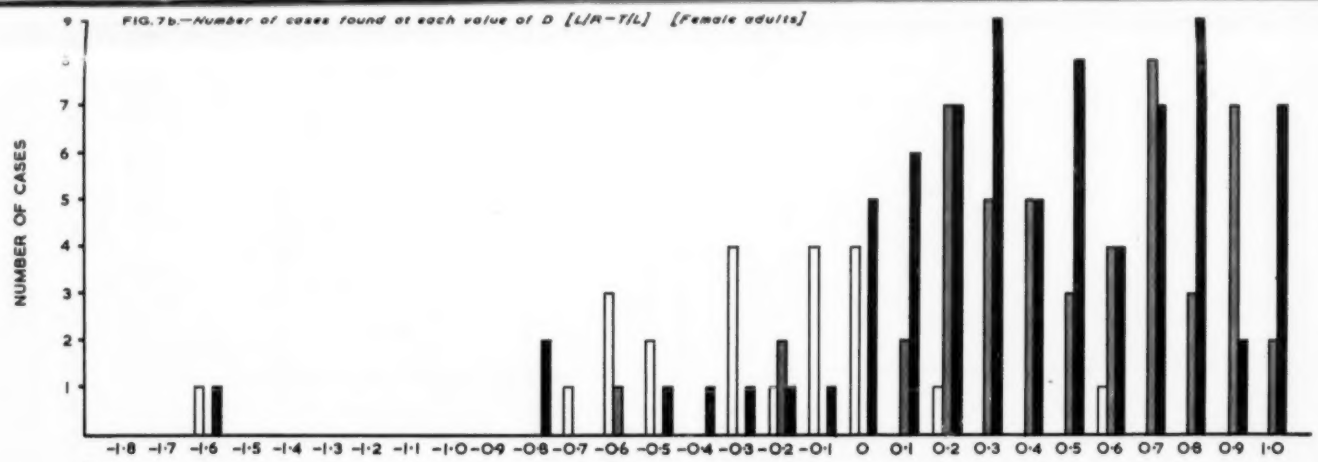
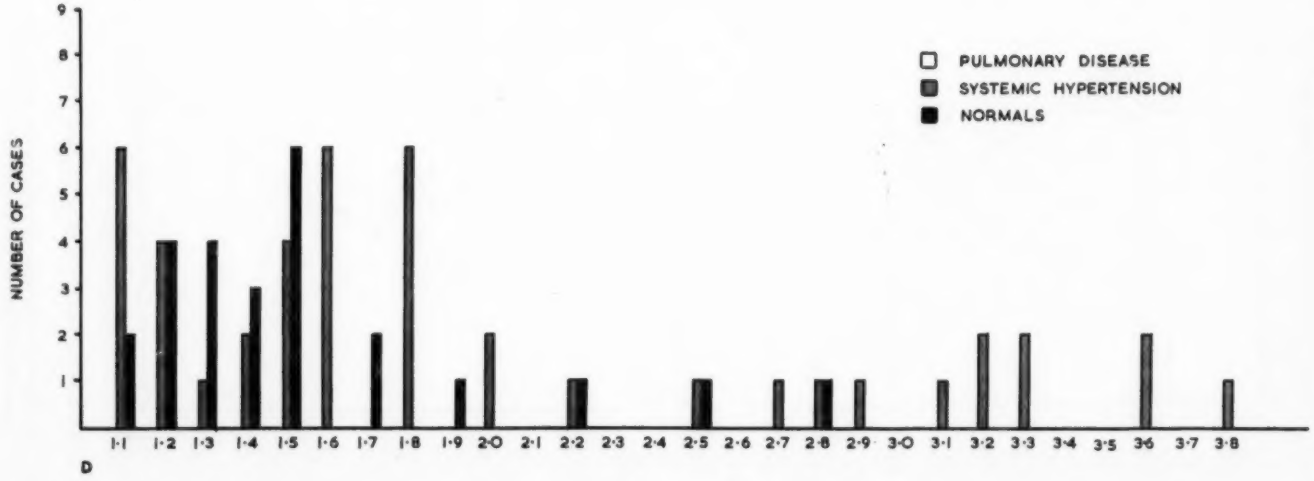


FIG. 7b.—*Number of cases found at each value of D [L/R−T/L] [Female adults]*



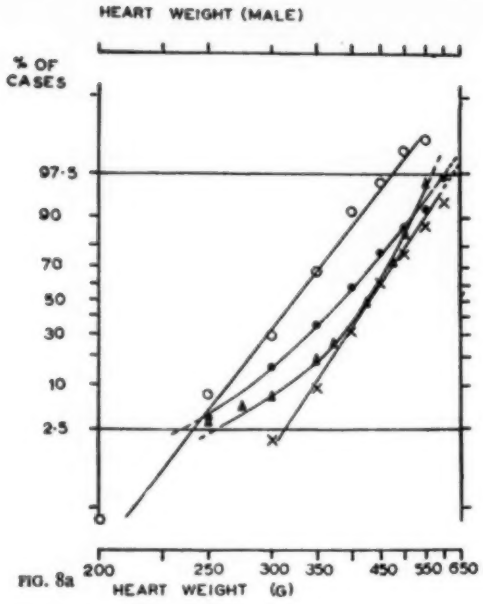


FIG. 8a

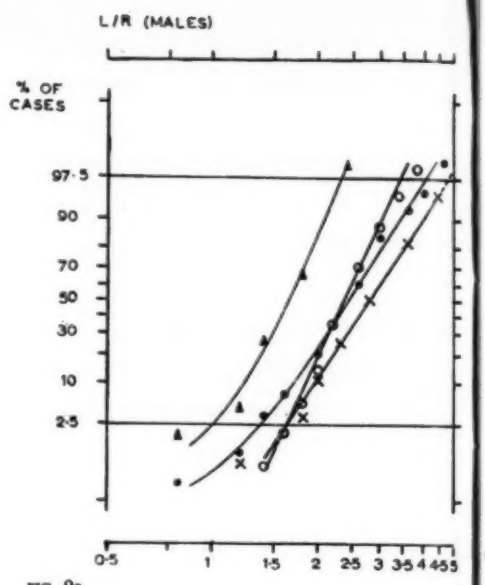


FIG. 9a

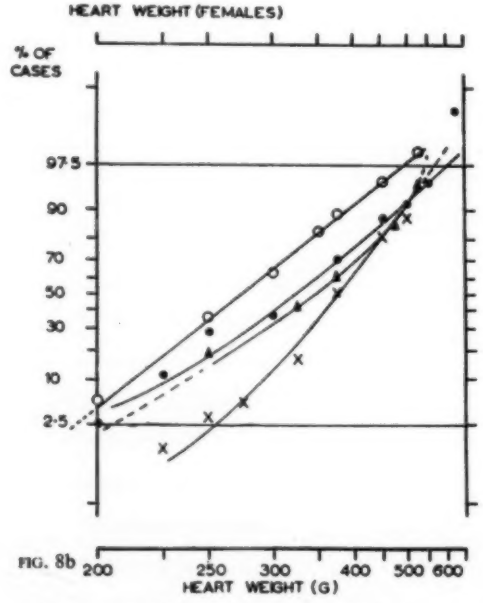


FIG. 8b

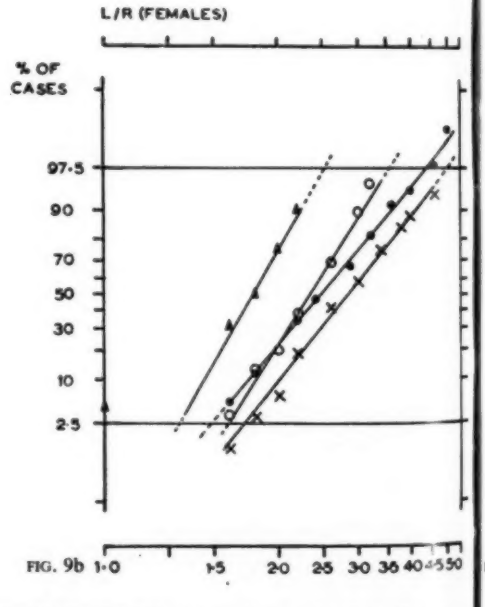


FIG. 9b

●——● = all cases, o——o = normals, ×——× = systemic hypertension, ▲——▲ = pulmonary disease

FIGS. 8a and b. *Percentage of cases found below each value of heart weight in each category (adults).*

FIGS. 9a and b. *Percentage of cases found below each value of L/R in each category (adults).*

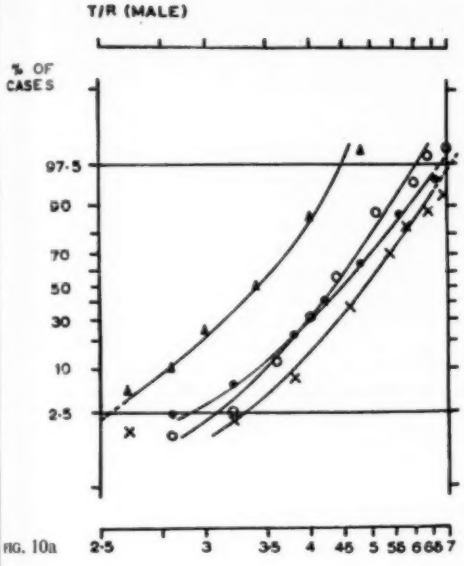


FIG. 10a

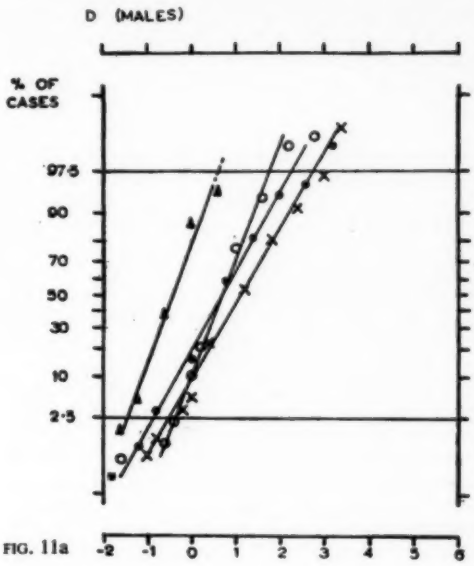


FIG. 11a

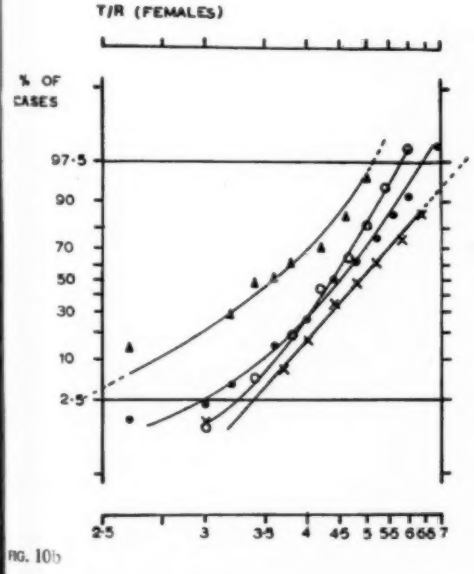


FIG. 10b

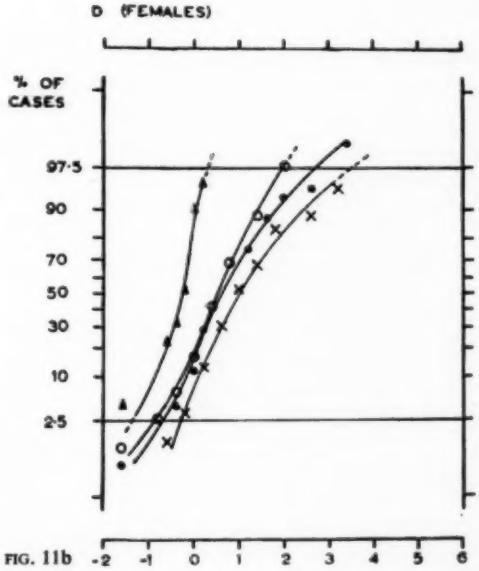


FIG. 11b

●—●—● = all cases, o——o = normals, ×——×——× = systemic hypertension, ▲——▲——▲ = pulmonary disease

FIGS. 10a and b. *Percentage of cases found below each value of T/R in each category (adults).*

FIGS. 11a and b. *Percentage of cases found below each value of D (L/R-T/L) in each category (adults).*

and may be associated with the interpretations which we have put on them. Although these calculations are approximate, there seems no reason to suppose that more laborious and exact methods would lead to different conclusions.

HEART WEIGHT ANALYSIS ACCORDING TO DISEASE Three diseases of the pulmonary system were extracted from Table I in order to determine if it is legitimate to include them in a category which is so suggestive of pulmonary hypertension.

Pulmonary tuberculosis There are seven cases in the 120 with reversed ratios and six cases in the 530 of normal or raised ratios. This difference is significant at the 1 in 1,000 level so that it seems justified to leave them as they stand in Table I.

Carcinoma of lung In the 120 cases with reversed ratios there are 10 cases of carcinoma of lung or bronchus and 25 in the 530 cases in the other groups. These differences are significant only at the 1 in 10 level and it is concluded that this disease is not associated with pulmonary hypertension. This material was therefore withdrawn from the pulmonary group and re-allocated to other categories; the new totals are shown in the final columns of Table I and these figures have been used in the other analyses.

Pulmonary embolism This is acute massive embolism usually post-operative or post-traumatic. There are 17 cases in the 120 with reversed ratios and 29 with normal or raised ratios. This difference being significant at the 1 in 1,000 level suggests that acute pulmonary embolism is associated with pulmonary hypertension and the two are bracketed in the final column of Table I.

If the figures in Table I are re-tabulated ignoring the total weight of the heart, much of their significance is lost, especially when the ratios are normal but less so when the ratios are raised or reversed. Perhaps this is due to including more cases of 'uniform hypertrophy' among the normals, but it destroys our hope that use of the fractions would entirely remove the difficulty of defining the weight of the normal heart.

HEART WEIGHTS IN ADOLESCENTS, CHILDREN, AND INFANTS An arbitrary division of this material into these three groups has been mentioned previously. Difficulty in deciding the normal heart weight in these cases (Sunderman and Boerner, 1949), lack of recorded blood pressure readings in almost all cases, and the small numbers prevent any valid analysis, especially in the first two groups. The findings in adolescents and children are shown in Table III

TABLE III

RESULTS IN ADOLESCENTS AND CHILDREN

	Male						*Female*					
	10 to 19 Years			*1 to 9 Years*			*10 to 19 Years*			*1 to 9 Years*		
	No. of Cases	*Age*		*No. of Cases*	*Age*		*No. of Cases*	*Age*		*No. of Cases*	*Age*	
Ratios normal	7	10	HW 95	6		No comment	5	11	HW 155 Bronchiectasis	6		No comment
		13	HW 260 Leukaemia					13	HW 195			
		13	HW 230 Appendicitis					13½	HW 235			
		16	HW 315 Bronchiectasis					14	HW 240			
		16	HW 235					15	HW 255			
		17	HW 290 Bronchiectasis									
		19	HW 280 Accident									
Ratios raised	4	10	Drowned, no details	5	2½	HW 70 Leukaemia	1	15	HW 215 Tuberculous meningitis	3	2½	HW 55 Septicaemia, lung abs congenital normal
		12	Accident, no details		3	HW 60 Leukaemia					5½	HW 130 B.P. 150 mm. Hg
		14	Cerebral haemorrhage B.P. 150/70 mm. Hg., unconscious		3	HW 85 Miliary tuberculosis					6	HW 130 Septicaemia adrenal haemorrhage B.P. read
		16	Rheumatic carditis		5	HW 85 Brain tumour						
					6	HW 220 Coronary thrombosis. No B.P. reading						
Ratios reversed	2	10	Mitral stenosis	1	6	Rheumatic carditis HW 100				3	2½	HW 80 Congenital heart dis
		14	Collapse, cor pulmonale								3½	HW 85 Fallot's tetralogy
											5	HW 100 Fibrocystic disease of pancreas and bronchiectasis

HW = Heart weight in grams

TABLE IV

RESULTS IN INFANTS

	Male		*Female*	
	No. of Cases		*No. of Cases*	
Ratios normal	18	All ages ½ hr. to 1 year, premature and stillbirth Two congenital heart disease, 3 days and 1 month One abnormal kidneys and ureters, 9 days No cases of hydrocephalus	11	All ages still birth to 5 weeks and premature One case hydrocephalus
Ratios raised	9	Two with abnormal kidneys and ureters, stillbirth and six weeks Two hydrocephalus, 4 months and 20 months	2	One intussusception, 11 weeks One hydrocephalus and spina bifida 10 weeks
Ratios reversed	31	Ratios equal (L/R = T/L) in six, 2 days to 2 months Two congenital disease of heart, 1 week to 6 weeks 23 all under 5 days except two at 14 and 12 days premature, one at 10 days no history, one at 26 days, hydrocephalus	24	Two congenital disease of heart 21 all under 5 days except one at 5 days hydrocephalus

and it will be seen that there is some suggestion that they are similar to those in adults. Two boys and one girl with normal ratios had bronchiectasis and it is probable that pulmonary hypertension with right ventricular hypertrophy had not yet become established at this age. Attention is drawn to the interesting possibility of systemic hypertension in two boys aged 14 and 6 years with raised ratios and also in two girls aged 5½ and 2½, the latter with renal disease. It will be further noted that two of the four cases with reversed ratios would be expected to have pulmonary hypertension.

The findings in 95 necropsies on infants are shown in Table IV and they suggest that the ratios, which are usually reversed at birth (as would be expected), begin to conform to the adult pattern at about a week or soon afterwards. Some possible reasons for a departure from this rule are also shown in Table IV.

The final conclusions may be re-stated simply: (1) When $L/R \geqslant 3$ and/or $T/R \geqslant 5$, there is left ventricular hypertrophy which, other causes being excluded, is suggestive of systemic hypertension. This association is enhanced if the weight of the heart is increased. (2) When L/R-T/L (D) is 0 or less, other causes being excluded, there is strong evidence of pulmonary hypertension. This association is largely independent of total heart weight and such values are unlikely to be found in the presence of cardiac hypertrophy consequent on systemic hypertension. Conversely it is most unlikely that pulmonary hypertension is present when $L/R \geqslant 3$ and/or $T/R \geqslant 5$.

DISCUSSION

It is important to stress that these figures can only be regarded as useful when they are considered with the whole of the clinical and post-mortem findings. In these circumstances a departure from normal ratios, whatever the weight of the heart, requires explanation and the method has therefore some value which is enhanced by its simplicity.

In the past, especially when there was no record of the blood pressure or when such a record might be regarded as unreliable owing to the clinical deterioration of the subject, there has been a tendency to consider an increased heart weight suggestive of systemic hypertension, other explanations being lacking. This new technique has to some extent defined more clearly the criteria on which such a conclusion could be sustained and has revealed a method by which pulmonary hypertension can be diagnosed after death.

The finding that acute pulmonary embolism is associated with pulmonary hypertension emphasizes the risk of post-operative and post-traumatic immobilization in patients with chronic pulmonary disease. Such people may be considered as especially suitable for early prophylactic anticoagulant therapy. Their extra susceptibility to this complication is presumably due to venous stasis consequent on the pulmonary hypertension and of course the added risk to such individuals is not confined to immobilization in hospital but is also present when they are confined to bed at home. Phear (1960), in a follow-up study of patients surviving pulmonary embolism, found that nearly 15% were severely disabled by pulmonary hypertension. It seems from our own findings that this condition was probably present before the embolic episode which in these severely disabled survivors may have accentuated it.

These results were compared with those published in detail by Lewis (1914) and by Herrmann and Wilson (1922) by calculating the ratios L/R, T/R, and T/L from their figures after adding the weight of the septum to their left ventricular weight. In the latter case, although the septum was divided, a reasonably good correlation was found with these conclusions, but in Lewis's tables no such correlation could be found. Herrmann and Wilson remark that

Lewis's method is inaccurate to the extent of 18 g. lost on one ventricle and calculations from his figures show that they deviate from these, chiefly in the ratios T/R and T/L.

It seems that the value of this method is enhanced by its applicability to either sex at any age and its apparent validity in children and infants should be of special interest.

I wish to acknowledge my gratitude to Professor J. R. Squire and members of his staff for their help with the statistical analyses and for some of the figures.

Dr. Avril Oatway has given much help both with necropsies and tabulating results. Other pathologists who performed necropsies are Dr. Martin Israel, Dr. Allan Jacobs, Dr. C. B. Whittaker, Dr. B. Godwin, Dr. H. G. Alton, Dr. Hameed.

Mr. H. J. Flewitt weighed and recorded all the material and we express our special thanks to him.

I wish also to thank Mr. A. Hemsley for drawing Figs. 4-7 inclusive, and Mr. R. Paton and Mrs. R. Harpin for the photographs.

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Radio-isotope investigations in primary myeloid metaplasia

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SYNOPSIS Chromium-51 and iron-59 studies in patients with primary myeloid metaplasia show that the anaemia of the condition is dependent on depression of marrow activity, aggravated in about one-third of cases by accelerated red cell destruction to which the marrow compensatory response is inadequate. Surface measurements of isotope activity assist in assessing dependence on extramedullary sites for erythropoiesis and in diagnosis. The relevance of these findings to the hypotheses of the pathogenesis of the condition is discussed.

The conditions which comprise the group known as the 'myeloproliferative disorders' are characterized by quantitative and qualitative abnormalities in the proliferation of haemopoietic cells for which no demonstrable cause is evident (Dameshek, 1951). It is not known at present whether any common aetiological factor unites the group: the essential basis for their being classified together lies in the abnormal proliferative tendency which extends to cells, such as the fibroblast and reticulum cell, which do not enter the peripheral blood. The various conditions differ not only in the combinations of cell-lines involved but in the degree of maturation of the most prominent cells affected, so that both acute and chronic forms of disorder may be distinguished.

The classical types of chronic myeloproliferative disease consist of polycythaemia vera, chronic myeloid leukaemia, essential thrombocythaemia, and primary myeloid metaplasia, while the acute forms include acute myeloblastic leukaemia, acute myeloid metaplasia, and the variants of the Di Guglielmo syndrome. As was strongly emphasized by Hutt, Pinniger, and Wetherley-Mein (1953), combinations of features frequently occur which overlap the conventional descriptions of these conditions, and the wide spectrum of clinico-pathological patterns includes many cases which lie between these categories. It is an essential part, however, of the study of disease to distinguish groups of cases showing common features, and to determine the significance of such groupings in terms of sequelae, prognosis, and pathological correlations. To be most useful, the criteria must be carefully selected, and difficulties have arisen from an undue emphasis on the peripheral blood findings in these diseases, since these may change radically during the course of a single case.

The development of satisfactory biopsy techniques has made histological criteria more useful clinically, and by such evidence it has proved possible to distinguish one such group of cases showing haemopoietic tissue in sites other than the bone marrow, especially in the spleen, and to a lesser extent also in the liver. Excluded from consideration are those which have predominantly blast-cell proliferation, the rare cases in which localized heterotopic marrow is found in isolation in sites such as the adrenal (Collins, 1932), and those in which extramedullary erythropoiesis is clearly compensatory to an anaemia of known origin. A confusing variety of names, summarized by Heller, Lewisohn, and Palin (1947), has been given to cases falling into this group, including myelosclerosis, megakaryocytic myelosis, aleukaemic myelosis, and panmyelosis. The broader term 'primary myeloid metaplasia' embraces all such cases regardless of the dominance of one or other cell-line, or the presence of variable features such as marrow fibrosis. It was introduced by Block and Jacobson (1950) following the use of 'agnogenic myeloid metaplasia of the spleen' by Jackson, Parker, and Lemon (1940) and Reich and Rumsey (1942), and the usage here differs only in not excluding cases which have a phase of polycythaemia.

The findings most commonly associated with myeloid metaplasia are a chronic anaemia of insidious onset, splenomegaly, a less prominent hepatomegaly, leucoerythroblastosis, and progressive fibrosis of the bone marrow. The haematological

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findings are variable and may include anaemia, polycythaemia, leucocytosis, leucopenia, thrombocytopenia, and thrombocythaemia. An account of the various clinical patterns of this condition has been given elsewhere (Bowdler and Prankerd, 1961). The predominant feature may be bone marrow failure with splenomegaly, polycythaemia, leucocytosis, thrombocythaemia or megakaryocytosis, or haemolytic anaemia, but the pattern may vary with time and the features become superimposed. Assessment of such cases may be difficult, especially when accelerated red cell destruction is present with marrow failure, and it was the purpose of this study to clarify aspects of red cell production and destruction in this condition by means of radio-isotope techniques, as has been done in chronic renal failure by Joske, McAlister, and Prankerd (1956) and by Bailey and Prankerd (1958) in thalassaemia.

METHODS

Chromium-51 labelled red cells and iron-59 tagged plasma were employed by combining the techniques described by Joske *et al.* (1956) and Harris, McAlister, Prankerd, and Singh (1957). A sample of the patient's red cells was labelled with 50 μc. of chromium-51 (as sodium chromate) and recombined with an approximately equal quantity of plasma treated with 5 μc. of iron-59, using ferric chloride of high specific activity. A small sample was set aside for later determination of activity by well counting and the remainder was injected intravenously into the patient after its activity in the syringe had been determined at a standard distance from the collimated scintillation counter used for the surface counting. The subsequent surface counts made over the lumbar spine, sternum, spleen, liver, and thigh at approximately daily intervals were expressed in fractions of the activity of the syringe count. By reason of the difference between the energies of the γ-radiations of the two isotopes, these could be assessed separately by the use of suitable selected settings of the discriminator bias. At the time of the successive surface counts, further blood samples were taken and their activity measured at the end of the investigation in a well counter.

From the results, the red cell life, iron utilization, and patterns of surface activity of the isotopes over the organs studied were calculated. Marrow activity was assessed by means of indices as described in the text. Methods of estimating erythropoietic activity which depend on the disappearance of iron-59 from the plasma have not been used in this study since they are open to theoretical objections in the presence of impaired iron utilization (Joske *et al.*, 1956) and have been shown to correlate poorly with other evidence of depressed red cell production by Nathan and Berlin (1959) in their cases of myeloid metaplasia.

In the course of the investigation it was found necessary to derive an expression for the iron-59 activity of an organ (as indicated by surface counting) which was arising from the blood within the organ and to distinguish this from the activity of isotope held in the tissues. This was possible where there was no accumulation of chromium-51 in the tissue or where this was negligible, by estimating the surface counts expected from the relative values of the activities of the two isotopes in the blood. The method is shown in detail in the Appendix.

CASES

Fourteen patients were investigated, three men and 11 women, between the ages of 26 and 74 years. The diagnosis rested on histological proof in 12 and details of these cases were included in the series described elsewhere (Bowdler and Prankerd, 1961). In the remaining two cases biopsy was not justified but the diagnosis was considered established on other grounds. In 13 cases a combined isotope investigation was made but in one only a chromium-51 study was performed. Details of the patients at the time of the investigation and a summary of the results are given in the Table. Eleven of the 14 patient had haemoglobin levels below 12·8 g. %.

TABLE

SUMMARY OF CLINICAL FEATURES AND RESULTS

Case	*Sex*	*Age*	*Hb (g.%)*	*WBC (per mm³)*	*Spleen Size (cm.)*	*T½Cr (days)*	*Iron Utilized (%)*	*T½Fe (days)*	*Marrow Index*	*Index of Marrow Activity*	*Iron-59 Surface Curves*	
											Marrow	*Spleen*
1 (1B)[1]	M	61	9·1	1,400	6	26	70	3·7	19	0·6	Low	Type 1
2 (1E)	M	64	12·6	11,200	3	17½	71	1·5	47	2·21	None	Type 1
3 (2A)	F	64	16·4	9,600	3	26	75	3·0	25	1·15	Low	Type 1
4 (2B)	F	75	12·5	24,000	12	26	50	2·6	19	0·85	Low	Type 1
5 (4A)	F	75	14·5	11,600	9	24	65	2·7	24	0·95	Normal	Type 1
6 (1A)	M	54	6·6	3,950	3	26	34	1·8	19	0·45	None	Type 2
7	F	63	10·2	4,200	14	26	59	2·3	26	0·68	None	Type 2
8	F	74	11·0	5,000	10	20	77	2·3	33	1·54	Low	Type 2
9 (5A)	F	66	3·6	1,400	RIF	6	66	2·4	28	2·63	Low	Type 2
10 (5C)	F	72	11·1	21,000	14	17	64	2·5	26	1·85	Low	Type 3
11 (1E)	F	55	12·0	2,600	12	24	75	3·1	24	0·80	Low	Type 3
12 (3A)	F	62	11·0	25,500	14	24	70	3·0	23	0·74	None	Type 3
13 (4C)	F	26	14·7	13,500	6	24	90	3·2	31	0·95	Low	Type 3
14 (5B)	F	50	3·4	6,200	12	14	—	—	—	0·85	—	—

[1]The case numbers in brackets relate to the clinical descriptions given by Bowdler and Prankerd (1961). This table gives the findings at the time of the isotope investigation

RESULTS

RED CELL SURVIVAL The orthodox method for the determination of red cell survival by the chromium-51 method was used, the results being expressed as the time for 50% of the chromium activity to disappear from the blood (=$T\frac{1}{2}Cr$). Normal values in this unit lie between 24 and 28 days. In nine cases survival was at or close to the normal value, and in five cases significantly reduced survival times were recorded, the $T\frac{1}{2}Cr$ in these being 20, 17·5, 17, 14, and six days respectively. Case 1 had a normal red cell survival on first being investigated but three years later he had become severely anaemic and was requiring frequent blood transfusions. At this time, the $T\frac{1}{2}Cr$ had fallen to nine days.

In Case 2, in which the $T\frac{1}{2}Cr$ of the patient's own cells was 17·5 days, a second study was made using fresh, normal, compatible donor blood. The donor red cell survival was not significantly different from that of the patient's cells ($T\frac{1}{2}Cr$ 17·7 days). In this subject, the only significant source of red cells detectable was the spleen. It was concluded that the haemolysis was not due to an intracorpuscular defect, and that spleen-derived red cells responded to the destructive agent to the same extent as normal cells originating from the marrow.

Surface counting over the spleen and liver for chromium-51 activity was used to assess the role of these organs in the destruction of red cells. The three cases with least reduction in red cell life showed no significant uptake of the isotope by these organs but in Cases 9 and 14 there was a distinct rise in the spleen-precordium ratio for chromium-51, with a lesser rise in the liver-precordium ratio. A similar pattern was found in Case 1 when the red cell life was reduced. In no case was the spleen involved alone.

IRON UTILIZATION Iron utilization is calculated as the product of the iron-59 activity of unit volume of red cells and the total red cell volume, which is obtained from the dilution of the chromium-51 labelled cells. The iron utilized on successive days is plotted as the 'iron utilization curve' (Finch, Gibson, Peacock, and Fluharty, 1949). From this curve the percentage utilization of the iron injected, which is normally over 90%, and the half-time for iron utilization (normal = 2·7 ± 0·66 days) can be found. The 'marrow index' proposed by Joske *et al.* (1956) as a measure of the net functional activity of the marrow in erythropoiesis has been calculated from the formula:

$$\text{Marrow index} = \frac{\text{percentage utilization } {}^{59}\text{Fe}}{T\frac{1}{2}\text{Fe}}$$

With one exception, iron utilization was impaired, the mean being 66·7 ± 13·6%. The exception was Case 13 which utilized 90% of the injected dose despite a reduced uptake of iron-59 by the lumbar spine. The mean $T\frac{1}{2}Fe$ for Cases 1 to 13 was 2·62 ± 0·59 days, which is not significantly different from normal, suggesting that there is a normal rate of maturation and release of the red cells. The mean marrow index in this series was 26·5 ± 8·1, which is significantly reduced.

The 'index of marrow activity' proposed by Chaplin and Mollison (1953) has also been calculated. This is equal to:

$$\frac{\text{Observed haematocrit}}{\text{Normal haematocrit}} \times \frac{\text{Normal red cell survival}}{\text{Observed red cell survival}}$$

and gives as a fraction of the normal the rate of red cell production required to maintain a steady packed cell volume. The value obtained for those cases without significant haemolysis (0·80 ± 0·21) was significantly less than that obtained for the cases with accelerated red cell destruction (1·82 ± 0·46) with $t = 4{\cdot}31$ and $P < 0{\cdot}01$.

LUMBAR SURFACE COUNTING The normal curve obtained by plotting the serial counts for iron-59 activity over the lumbar spine is shown in Fig. 1A. In five cases no activity could be demonstrated, in seven cases the activity was depressed, and in only one was a normal curve obtained. The activity expressed in these curves depends on three factors: The geometry of the marrow space, which is approximately constant between subject and subject; the unit activity of the marrow within the space, which is the main variable; and the total marrow volume which determines the fraction of the active iron presented to the marrow. This last factor leads to a reduction in the height of the uptake curve with expansion of the marrow volume, as in polycythaemia vera. At present a satisfactory method of correcting for the effect of variable marrow volume is lacking but it is probable that the marrow volume is reduced in the cases studied here and the curves underestimate the depression in activity.

SPLENIC SURFACE COUNTING Three distinct patterns were observed in surface counting for iron-59 over the spleen.

Type 1 showed maximal activity on the first day after injection and thereafter the activity declined rapidly until five to seven days later. This is shown in Fig. 2B, which illustrates the similarity of this curve to that found by counting over normal marrow, as in Fig. 1A. Five cases showed this pattern.

Type 2 showed a continual smooth rise in activity as in curve A in Fig. 2A. The maximum height was

FIG. 1A

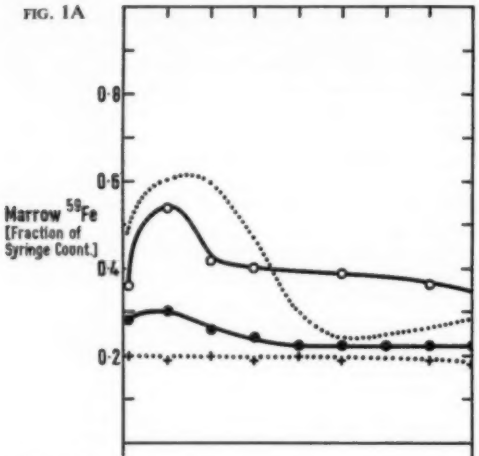

FIG. 1B

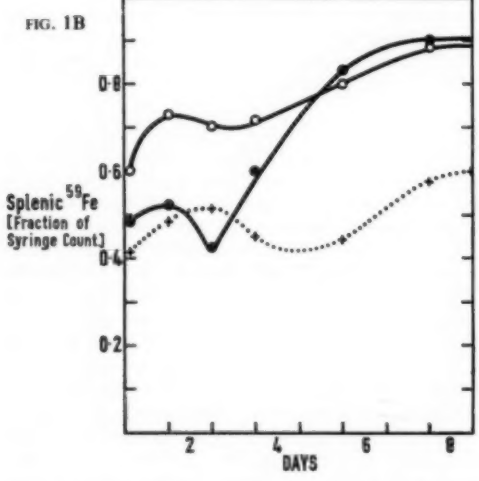

FIG. 1A. *Graph of marrow iron-59 activity plotted against time. Case 1* (●——●——●) *and Case 10* (o———o) *illustrate depressed marrow activity and no activity is detectable in Case 2* (+····+····+).

FIG. 1B. *Graph of the splenic iron-59 activity plotted against time, showing examples of the type 3 pattern. Case 10* (+·····+), *Case 11* (●———●), *and Case 12* (o———o).

FIG. 2A

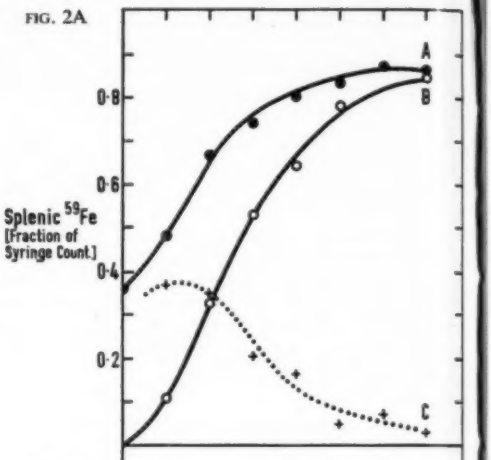

FIG. 2B

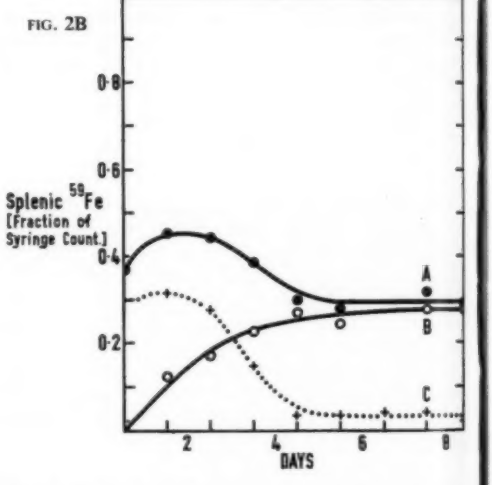

Fig. 2A. Graph of splenic iron-59 activity plotted against time, showing the type 2 pattern. Observed activity A (●———●), *calculated blood activity in the organ B* (o———o), *and the difference between the activities C* (+·····+).

FIG. 2B. *Graph of splenic iron-59 activity plotted against time to illustrate the type 1 pattern (symbols as for Fig. 2A).*

reached between the fourth and eighth days and was followed by a sustained high plateau of activity. Four cases showed this pattern.

Type 3 showed a biphasic curve with an initial peak on the first day, followed after a shallow trough by a further rise, usually exceeding the first. Four cases showed this pattern and examples are shown in Fig. 1B.

The type 1 and type 2 curves are shown in Figs. 2B and 2A, together with the estimated curves for the activity of the blood in circulation through the organ. The differences between the observed and the

calculated curves are also plotted to give patterns which strongly resemble the normal iron uptake and release curves of bone marrow. These reflect the passage of iron through the splenic erythropoietic tissue and provide the principal component of the type 1 curve. In the type 2 curve the most prominent component is due to the circulating iron activity, while both components are distinguishable in the type 3 curve.

HEPATIC SURFACE COUNTING Changes in the iron-59 surface counts over the liver seldom gave clear evidence of intrahepatic erythropoiesis, no doubt due to the relative sparseness of the haemopoietic elements in this organ in most cases. Cases 3 and 13, however, showed a suggestive rise and fall in the counting rates, but this was of much lower magnitude than was found over the spleen in the type 1 pattern.

THIGH SURFACE COUNTING This was performed over the lower third of the thigh to detect the presence of marrow extension into the distal femur. This was demonstrated in Case 12 despite the absence of any demonstrable activity in the lumbar spine.

DISCUSSION

The anaemia of primary myeloid metaplasia thus appears to depend on impaired marrow activity and in some cases on accelerated red cell destruction. The shortened red cell life was demonstrated in the cases described by Weinstein and LeRoy (1953), Nathan and Berlin (1959), and Desforges, Ross, and Moloney (1960), but in the longer series of Fudenberg and Mahoney (1958) the incidence of significant haemolysis was closer to that found here. It is probable that the later in the course of the disease the investigation is made, the greater the likelihood of finding a shortened red cell life. Fudenberg and Mahoney (1958) have produced evidence from the survival times of red cells cross-transfused between normal subjects and patients with myeloid metaplasia that both intra- and extracorpuscular factors may be responsible for the haemolytic tendency; it is clear from Case 2 of this series that in certain cases at least the extracorpuscular factors may be predominant. In those cases in which significant organic uptake of chromium-51 accompanied erythrocyte destruction, the spleen, while showing the most marked accumulation of the isotope, was not involved in isolation. The appreciable degree of accumulation in the liver demonstrates that there is an extrasplenic element, which is suggestive of a metabolic defect in the red cells (Harris, McAlister, and Frankerd, 1957). This would account for the variable success of splenectomy in alleviating the premature destruction of red cells in this condition.

Following the description of cases under the title of 'agnogenic myeloid metaplasia' by Jackson *et al.* (1940), it was suggested that a possible explanation of the extramedullary haemopoiesis was that it arose in compensation for haemolysis. Thus, Claman and Collier (1952) described the case of a young adult with an acute haemolytic anaemia, in whom well-marked haemopoiesis was demonstrable in the spleen. They concluded that 'the diagnosis of agnogenic myeloid metaplasia is rarely, if ever, tenable. At best it is a misnomer.' It is clear, however, from Case 1 that myeloid metaplasia may be demonstrated long before red cell destruction is significantly accelerated, and there are no grounds for supposing that haemolysis played any part in initiating the metaplastic process.

The depression of marrow activity commonly found in this condition is reflected in the impaired iron utilization, and in the low values obtained for the marrow index (Joske) and the index of marrow activity in this series. None of these patients, however, was examined while in the polycythaemic state which may accompany the condition. There was also failure of the erythropoietic tissue to respond adequately to the additional demands imposed by a shortened red cell life. It has been shown by Crosby and Akeroyd (1952) that under conditions of long-standing haemolysis the normal bone marrow can compensate for a red cell life as low as 15 days, with a rate of haemoglobin production about seven times the normal. In Cases 2, 8, and 10 the mean red cell life was between 40 and 50 days but all three patients were anaemic, and of the five with haemolysis the greatest response found was an increase of red cell production to 2·63 times normal.

The view has been frequently expressed that myeloid metaplasia arises in compensation for the loss of bone marrow activity resulting from marrow sclerosis or atrophy (Donhauser, 1908; Stephens, and Bredeck, 1933; Chapman, 1933; Wolf, 1932; Jordan and Scott, 1941; Leonard, Israels, and Wilkinson, 1957). In Case 2, the only detectable source of red cell production was the spleen, and the 'marrow' activity, as shown by both indices, was higher than normal. Nevertheless, the spleen was not greatly enlarged, and it is clear that the massive splenomegaly frequently encountered in these cases is greatly in excess of that required to accommodate a compensatory volume of haemopoietic cells. Conversely, the limited response to the additional requirements imposed by haemolysis does not seem to depend on spatial restriction, even in the presence of sclerotic obliteration of the marrow cavities, since anaemia may arise before the considerable potential for splenic enlargement is exhausted. It is

also apparent from Case 5 that the myeloid metaplasia demonstrated by biopsy preceded significant bone marrow impairment; the iron-59 surface counting curve for the lumbar spine was normal, and since the marrow sclerosis in this condition usually proceeds centrifugally it is unlikely that marrow obliteration had advanced far in this case.

The leucoerythroblastosis shown by many patients with this condition has been ascribed to the extramedullary sites lacking the normal regulatory mechanisms for cellular release (Wintrobe, 1951; Leonard *et al.*, 1957; Hayhoe and Whitby, 1955). However, the removal of the most active site of metaplastic haemopoiesis by splenectomy is frequently followed by the persistence of immature cells in the blood, as shown by the cases of Green, Conley, Ashburn, and Peters (1953). Neither the release phase of the splenic iron-59 curves nor the normal half-time for iron utilization suggested premature release of haemoglobinized cells.

With regard to the megakaryocytosis found in extramedullary sites in this condition, the view has been expressed that it contributes little to the appearance of platelets in the blood stream (Schwarz, 1954). Such doubts do not appear to apply to the erythropoietic elements, since the pattern of iron uptake and release over the spleen corresponds closely to that found over normal bone marrow in those cases with type 1 curves. These have also been described by Lawrence, Berlin, and Huff (1953) and by Schwartz, Tendler, Sharney, and Wasserman (1958). Curves similar to the type 2 pattern have been described by Huff, Elmlinger, Garcia, Oda, Cockrell, and Lawrence (1951), Schwartz *et al.* (1958), and Desforges *et al.* (1960), and have been ascribed to the reaccumulation of iron-59 containing cells in the spleen either immediately after their production or after release into the blood stream. However, in the present series, it has been shown that such a curve may be present without a reduction in red cell life, and that it arises principally from the activity of blood circulating through the organ.

The pathogenesis of primary myeloid metaplasia is still controversial. The present findings are consistent with the hypothesis put forward by Vaughan and Harrison (1939) that cells of mesenchymal origin are affected by a stimulus to proliferation, the differing patterns resulting from the degree to which the various cell lines are affected. This leads to haemopoietic cells, reticulum cells, and fibroblasts proliferating not only in the marrow cavities but in extramedullary sites as well. This would appear to be accompanied by a lessening of the responsiveness of the erythropoietic series to other stimuli, and it may be that in the later stages a competitive process limits the erythropoietic cell mass in favour of the fibroblast.

It remains to consider the value of radio-isotope studies in the management of these cases. First, they are useful in the detection of haemolysis, since the usual clinical tests may fail in the absence of a hyperactive marrow. Secondly, there appears to be a place for splenectomy in this condition, especially in the presence of severe haemolysis with a high transfusion requirement (Cartwright, Finch, Loeb, Moore, Singer, and Dameshek, 1955) and surface counting over the spleen provides some evidence of chromium-51 accumulation in this organ, which may be compared with other sites. In addition, Hickling (1937) has drawn attention to the hazard of removing the spleen when it is a major source of blood formation, and, although the basis for his contentions has been criticized (Green *et al.*, 1953), it remains true that some cases are indeed very dependent on the extramedullary sites for haemopoiesis and in these splenectomy would be ill advised. Isotope studies provide useful information on the residual activity of the bone marrow and assist in assessing the dependence of the subject on the metaplastic sites. Lastly, there is a small group of cases in which biopsy of liver, spleen, or bone is inadvisable owing to a haemorrhagic tendency, and in these isotope techniques may offer an alternative method of diagnosis.

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SUMMARY

A series of patients with primary myeloid metaplasia has been investigated by radio-isotope techniques using chromium-51 and iron-59 to assess red cell survival, iron utilization, and the patterns of the surface counts over various organs.

Bone marrow activity assessed by the counting for iron-59 over the lumbar spine was usually depressed and iron utilization was usually impaired. Severe anaemia in one subject arose from reduction in erythropoiesis alone.

Red cell destruction was accelerated to a significant degree in five of the 14 patients and was shown to develop as a late event after the onset of the condition in one further case. The erythropoietic response to the haemolytic process was shown to be subnormal.

Iron-59 counting over the spleen showed a cycle of

iron uptake and release closely corresponding to the pattern of normal marrow in some cases. In other cases, the iron-59 activity of the circulating blood was prominently reflected in the surface counts and a method of analysing such patterns is given. Certain cases show a marked dependence on the extramedullary sites of blood formation.

Within the limits of these investigations, the extramedullary erythropoietic cycle has not been shown to differ significantly from normal, and in one instance it was possible to show that the red cells produced responded in the same degree as normal cells to an extracorpuscular haemolytic process.

The spleen was not found to be the isolated site of red cell destruction in any of the present cases, although it might be the most active organ in this respect.

The value of this type of investigation in the management and diagnosis of this condition has been discussed.

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APPENDIX

It was necessary in this investigation to derive an expression for the iron-59 activity of an organ, with special reference to the spleen, as distinguished from that of the blood flowing through it. In order to allow for possible changes in the blood content of the organ with time, the correction was based on the ratios of the two isotopes, chromium-51 and iron-59, as measured in the blood, and the relative counting rates of the isotopes over the organ concerned. Cases in which there was a demonstrable accumulation of chromium-51 in the organ were excluded from this method.

The isotopes within an organ will be partially screened by the thickness of tissue lying between them and the surface counter. Owing to the lower energy of the gamma radiation of chromium-51, the attenuating (Compton) effect of the tissues will be greater for this isotope than for iron-59. Hence the recorded counting rates will be more greatly reduced. The screening effect will also differ between subjects and must be calculated for cases individually.

For screened irradiation of a given energy, $I = k.I_o$, where I is the emergent radiation, I_o is the energy of the source, and k is a constant dependent on the energy of the radiation, the partial absorption coefficient of the screening material and the thickness of the screen. In the case of an organ such as the spleen, the value of k will differ for the two isotopes but for each isotope will be constant over limited periods for a given subject. Thus:

$$S'_{te} = k_{te} \cdot S_{te}$$
$$\text{and } S'_{cr} = k_{cr} \cdot S_{cr}$$

where S'_{te} and S'_{cr} are the activities of the isotopes recorded by surface counting, S_{te} and S_{cr} are the source activities of the blood within the organ, and k_{te} and k_{cr} are constants dependent on the thickness and density of the screening tissues.

The source intensity will be proportional to the activities of the isotopes in the blood given by well counting, and thus:

$$S_{te} = k'_{te} \cdot W_{te}$$
$$\text{and } S_{cr} = k'_{cr} \cdot W_{cr},$$

where W_{te} and W_{cr} are the activities recorded on well counting, and k'_{te} and k'_{cr} are constants depending on the apparatus and the geometry of the organ. It follows therefore that:

$$S'_{te} = k_{te} \cdot k'_{te} \cdot W_{te}$$
$$\text{and } S'_{cr} = k_{cr} \cdot k'_{cr} \cdot W_{cr}$$

$$\text{Thus, if } k'' = \frac{k_{te} \cdot k'_{te}}{k_{cr} \cdot k'_{cr}}, \text{ then } \frac{S'_{te}}{S'_{cr}} = k'' \cdot \frac{W_{te}}{W_{cr}}$$

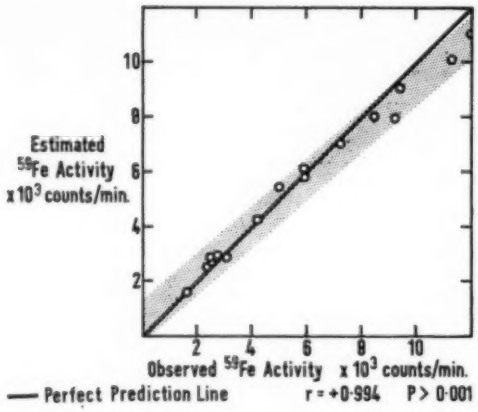

FIG. 3. *Graph of estimated iron-59 activity of a model spleen against measured activity. The shaded area lies between the 95% confidence limits of the points shown.*

it is possible to determine S'_{te}, S'_{cr}, W_{te}, and W_{cr} from the initial counting over the organ after allowing time for the injected blood to mix with the circulation, and from the blood activity of the isotopes at this time. Therefore k'' can be found, and for subsequent days the activity of the iron-59 in the blood within the organ calculated. This is corrected as usual for decay from the day of injection and expressed as a fraction of the syringe count.

To test the reliability of the method in predicting the organ activity of one isotope from the activity of the second and the blood values, observations were made over a model spleen, consisting of a wax shell made as a cast of an enlarged spleen removed at necropsy and having a volume of about 1,600 ml. The model was filled with water and the two isotopes were added, the concentrations being varied independently. The surface recordable activity of the model and the activities of the isotopes it contained were measured, and the iron-59 activity of the model was predicted by the method outlined. The results are plotted in Fig. 3 and the correlation between the measured and the predicted activity was considered satisfactory.

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The tongue and oesophagus in iron-deficiency anaemia and the effect of iron therapy

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SYNPOSIS Biopsies of the tongue and oesophagus were performed on 14 patients with uncomplicated iron-deficiency anaemia before and after treatment with iron. Haemoglobin and serum iron estimations were performed at the same time.

Nine patients had clinical evidence of atrophic changes in the tongue before therapy was started. Evidence of regeneration appeared within one or two weeks of starting iron therapy. Two patients showed persistent atrophy. Angular stomatitis and koilonychia were longer in disappearing.

Biopsies confirmed that filiform papillae and kerato-hyalin granules are frequently absent from the epithelium of the smooth tongues of iron-deficient patients. Iron therapy is followed by the re-appearance of keratohyalin granules and keratinized filiform papillae.

Two patients complained of dysphagia, which disappeared after treatment. No abnormality in the oesophageal epithelium was found in any of the patients either before or after therapy. The relationship of oesophageal carcinoma to antecedent iron-deficiency epithelial changes is considered suspect.

The association of atrophy of the mucosa of the tongue and dysphagia with anaemia has been recognized for many years. The nature and relative importance of the types of deficiency responsible for this syndrome have remained obscure. Deficiency of iron, of intrinsic factor, or of other vitamins has been implicated. Even when it had become clear that glossal atrophy occurred in primary iron-deficiency anaemia, it was considered that this could be due to secondary bacterial infection or to associated vitamin deficiencies.

Dysphagia associated with iron-deficiency anaemia was described independently by Kelly (1919) and by Paterson (1919) in papers entitled respectively 'Spasm at the entrance of the oesophagus' and 'A clinical type of dysphagia'. Vinson (1922) described 69 cases in a paper entitled 'Hysterical dysphagia'. The nature of the relationship between the dysphagia and the iron deficiency state was not clearly appreciated and the dysphagia was attributed to syphilis, to neurological, or to psychological disorders.

Suzman (1933), however, described marked improvement in the oral lesions and dysphagia in six out of seven cases after treatment with iron. Waldenström (1938) also treated three cases with iron, and concluded: 'Under the influence of iron therapy the epithelial defects in the corners of the mouth, on the tongue, and in the throat are healed'. Scant attention has been paid to the pathology of the lingual and oesophageal lesions associated with iron-deficiency anaemia. Savilahti (1946) gave an account of the findings in an old woman with the Paterson-Kelly syndrome who died with pneumonia. The tongue showed no filiform papillae, and only a few fungiform, and the whole epithelium appeared atrophic. The oesophageal and laryngeal epithelium was described as degenerated and atrophic but with a hyperplastic basal-cell layer. An area of muscle atrophy was also described in the middle third of the oesophagus; this was thought to explain the dysphagia. Cheli, Dodero, Celle, and Vassalotti (1959) found no relation between clinical depapillation of the tongue and histological atrophy. Jacobs (1960) found that the thickness of the buccal epithelium was reduced in some anaemic patients but that the mean epithelial thickness of anaemic patients as a whole was not significantly reduced.

The present study correlates the haemoglobin and serum-iron levels with the clinical and histological

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state of the lingual and oesophageal mucosa in patients with uncomplicated iron-deficiency anaemia, before and after treatment with iron.

METHOD OF STUDY

Fourteen patients (two male, 12 female) with iron-deficiency anaemia were selected. They all fulfilled the following conditions: (1) the initial haemoglobin level did not exceed 10 g./100 ml.; (2) blood or marrow findings were consistent with the diagnosis; (3) the serum iron was not more than 50 μg. per 100 ml.; (4) no iron therapy had been given for three months; (5) there was no clinical evidence of vitamin deficiency; (6) there was no history of recent blood loss, apart from menstruation; (7) renal disease, chronic infection, ulcerative colitis, rheumatoid arthritis, and neoplastic disease were excluded.

The first biopsies of tongue and oesophagus were taken within a week of the initial blood count. The patients then began treatment with iron, either oral ferrous gluconate, 2 g. daily, or an equivalent amount of ferrous glycine sulphate (intramuscular iron had to be given to one patient). The second biopsies were taken four weeks, and the third at three months after the start of therapy. Blood counts and serum iron estimations were performed on both these occasions.

BIOPSY TECHNIQUES

TONGUE The biopsy tube described by Taft, Hughes, and Wood (1958) is used. This consists of a knife 11 mm. long and 4 mm. wide mounted on one end of a stainless steel tube and covered with a stainless steel head in which there is a hole 2 mm. in diameter through which the biopsy is taken. The patient lies with his head supported by a pillow and, with the mouth open widely, protrudes the tongue. Wood and his colleagues anaesthetized the selected area with a submucosal injection of 2% procaine but as the biopsy is virtually painless we have found this unnecessary; an amethocaine lozenge sucked some 10 minutes before the biopsy provides adequate anaesthesia. The biopsy tube is applied lightly to the tongue with the lateral hole over the selected area and negative pressure sharply applied by an assistant by means of a 20 ml. syringe attached to the lateral tube. The knife blade is then quickly pulled down across the hole. The head of the biopsy tube is then removed and the specimen placed in 10% formol saline.

With this technique 70 biopsies have been attempted and a suitable specimen has been obtained in all cases, though with difficulty in some atrophic tongues. To obtain good results we have found it important to check that the knife blade is both sharp and correctly adjusted before biopsy. Bleeding from the biopsy site is negligible and ceases in two or three minutes. The biopsy site heals in every case without residual ulceration.

OESOPHAGUS It is assumed that the upper end of the oesophagus is the part most likely to show histological changes in anaemia, as it is this area to which the symptom of dysphagia is usually referred and in which the post-cricoid web occurs. The fact that mediastinitis can be a complication of trauma to the lower two-thirds of the oesophagus was another factor which persuaded us to confine our attention to the upper end of the oesophagus, which begins at about the level of the sixth cervical vertebra.

The patient fasts for 12 hours and sucks an amethocaine lozenge 10 minutes before the biopsy. The biopsy is taken with the gastric biopsy tube described by Wood (Joske, Finckh, and Wood, 1955). The metal tip, smeared with liquid paraffin, is placed on the back of the tongue and swallowed until the tip is approximately at the level of the sixth cervical vertebra. Radiological control in earlier cases confirmed the tube to be in position when the tip was 7 in. behind the incisor teeth and in subsequent biopsies we have used this measurement to confirm the tube to be in the correct position. The patient is then told to stop swallowing and while negative pressure is applied by an assistant the knife blade is quickly pulled down and a biopsy taken. The specimen is immediately placed in 10% formol saline.

Over 50 oesophageal biopsies have now been attempted and a specimen obtained on each occasion. The patient suffers no discomfort other than that caused by the presence of the tube and an occasional slight dysphagia of no more than 48 hours' duration. Most patients have been kept under observation for at least 24 hours after biopsy and none have developed mediastinitis, haematemesis, melaena, or other complication. Specimens of faeces have been examined for occult blood in several normal controls with negative results. The subject is able to enjoy a meal within two or three hours of the biopsy without any discomfort.

Occasionally the knife blade grips the mucosa without severing it completely. It is necessary to remove the tube slowly in case this occurs, to avoid excessive trauma. If the tube is found to be sticking, the knife blade must be released and a further attempt made.

CLINICAL OBSERVATIONS

All 14 patients described a rapid subjective improvement, *i.e.*, decreased lassitude and sore tongue, within a week of starting iron therapy, comparable with the subjective improvement seen after cyanocobalamin in pernicious anaemia. The signs of repapillation of the tongue, where atrophic, appeared after one to two weeks' treatment, and the tongue was fully papillated in 12 patients at the time of the third biopsy. A normal tongue with distinct papillae was easily recognizable. Complete atrophy with loss of any distinctive papillae was either total or localized to the central or peripheral zones of the tongue. After one to two weeks' iron treatment small, regenerating papillae could be seen and these have been termed 'stubby papillae'.

The rapid regeneration of papillae in the tongue contrasted with the slower disappearance of angular stomatitis. This was often still present at the time

of the second biopsy, when the tongue was normal or showing early repapillation. Koilonychia took even longer to grow out and was present in the distal half of the nail at the time of the third biopsy after three months' iron therapy. It is concluded that regeneration of lingual papillae is one of the most sensitive indices of response to iron.

Two patients showed persistent glossal atrophy after three months' therapy. In Case 9 the haemoglobin and serum iron were normal but peripheral atrophy of the tongue persisted, whereas in Case 14 there was generalized atrophy in spite of a haemoglobin of 13·4 g.%.

Two patients with anaemia had an associated dysphagia. Case 13 presented symptoms of sore tongue and dysphagia for 12 months, with a moderate anaemia (initial Hb of 7·4 g.%) and a serum Fe level of 12 μg./100 ml. Angular stomatitis was also present. Rapid repapillation of the tongue occurred within seven to 14 days and the dysphagia had disappeared within seven days. However, by the time of the third biopsy the tongue was again sore and atrophic but without dysphagia; the haemoglobin and serum iron levels were then normal.

Case 14 had symptoms of sore tongue and dysphagia for 18 months. She was found to have 'stubby papillae', angular stomatitis, and koilonychia. The dysphagia was slower to respond and was present until eight weeks after starting oral iron. The tongue was fully papillated two months after therapy was begun. The two patients with anaemia and dysphagia had the lowest serum iron levels of the series (12 and 10 μg./100 ml. respectively), but satisfactory treatment of both anaemia and dysphagia was achieved.

In one patient only was the response to oral iron unsatisfactory (Case 4), and a subsequent follow-up of this patient showed a chronic duodenal ulcer, which required gastrectomy.

HISTOLOGICAL OBSERVATIONS

NORMAL APPEARANCES The following description is based on the appearances of biopsies in normal subjects. The biopsies of the tongue yielded a piece of epithelium of 2 to 3 cm. diameter. The depth of the biopsy ranged from 0·8 to 1·0 mm. On only one occasion did the biopsy fail to include the full thickness of the epthelium.

The biopsies of the oesophagus provided slightly larger specimens. The diameter of the epithelium removed ranged from 2 to 4 mm., with a depth of 0·8 to 1·4 mm. Apart from a single failure, all the biopsies included the full thickness of the epithelium.

The specimens were fixed immediately in formol saline. Orientation of the specimen for subsequent sectioning is made easier if a little methylene blue is added to the fixative just before its transfer to alcohol. Alternatively, picric acid can be used, or the specimens fixed in Bouin's fluid.

The lingual epithelium, as seen in these biopsies, varied from 15 to 40 cells in thickness. The epithelium shows alternating areas of swollen, glycogen-filled cells, and of more compact cells with more solid-looking cytoplasm. These latter areas underlie the filiform papillae, which appear as slender, stalked projections covered at their tips by a dense layer of microorganisms. The compact epithelial cells deep to the papillae show a scattering of keratohyalin (eleidin) granules. A narrow strip of the submucosal connective tissue is included in nearly all the biopsies, but no muscle fibres or salivary tissue were encountered. A typical lingual biopsy specimen taken from a healthy control is shown in Fig. 1.

The oesophageal epithelium has a thickness of 15 to 35 cells. There is a smooth transition from the columnar cells of the basal layer to the flattened cells, with their elongated pyknotic nuclei, of the superficial layers. There is no variation in the pattern such as is seen in the tongue, and the superficial layers form a smooth surface. The biopsies included part of the submucosa, and in many cases bundles of smooth muscle from the muscularis mucosae; sometimes the duct of a mucous gland, with its accompanying sheath of lymphocytes, was also included.

Fig. 2 shows the greater part of an oesophageal biopsy taken from a normal control showing the features mentioned.

APPEARANCES IN IRON DEFICIENCY AND CHANGES AFTER IRON THERAPY The *tongue* was normal in five patients and showed some abnormality in nine patients before treatment. In the biopsies of the nine clinically smooth tongues taken before the start of iron therapy, the epithelium showed no evidence of filiform papillae and no keratohyalin granules were present (Fig. 3). With restoration of the haemoglobin and serum iron to normal, the tongue biopsies revealed the reappearance of filiform papillae in most cases. Accompanying the papillae, scattered groups of keratohyalin granules reappeared in the underlying epithelial cells (Fig. 4). These granules only occurred at the sites where filiform papillae had formed. They were scattered throughout the depth of the Malpighian layer of the epithelium (Fig. 5). There was no correlation between the number of cell layers and the clinical and haematological state, nor was there any evidence of increased mitotic activity following iron therapy. No evidence of keratosis or parakeratosis was found.

FIG. 1

FIG. 1. *Lingual biopsy from a healthy control. Haematoxylin and eosin* (× 50).

FIG. 2. *Oesophageal biopsy showing normal epithelium, and the duct of a mucous gland with its lymphocyte sheath. Haematoxylin and eosin* (× 50).

FIG. 2

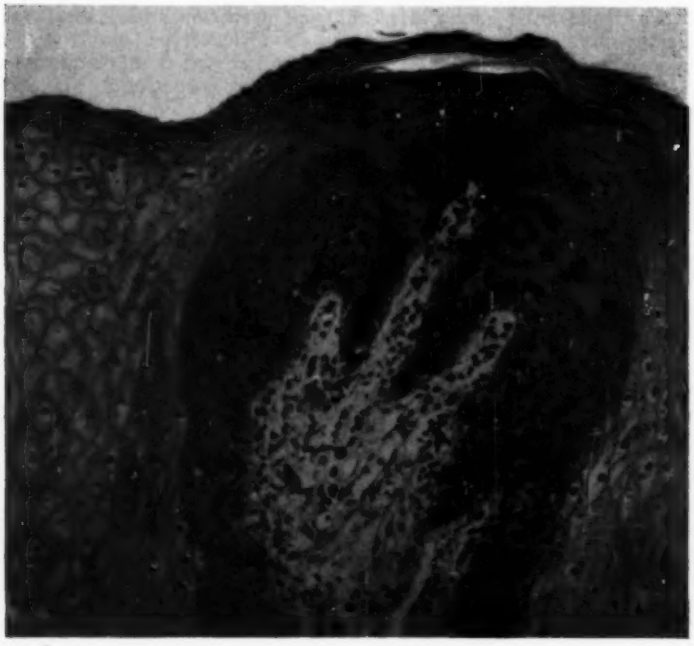

FIG. 3

FIG. 3. *Lingual biopsy showing atrophy of filiform papillae in a smooth tongue before therapy. Haematoxylin and eosin* (× 210).

FIG. 4. *Lingual biopsy showing reappearance of filiform papilla and keratohyalin granules. Haematoxylin and eosin* (× 210).

FIG. 5. *High-power view showing keratohyalin granules at the base of a filiform papilla. Haematoxylin and eosin* (× 600).

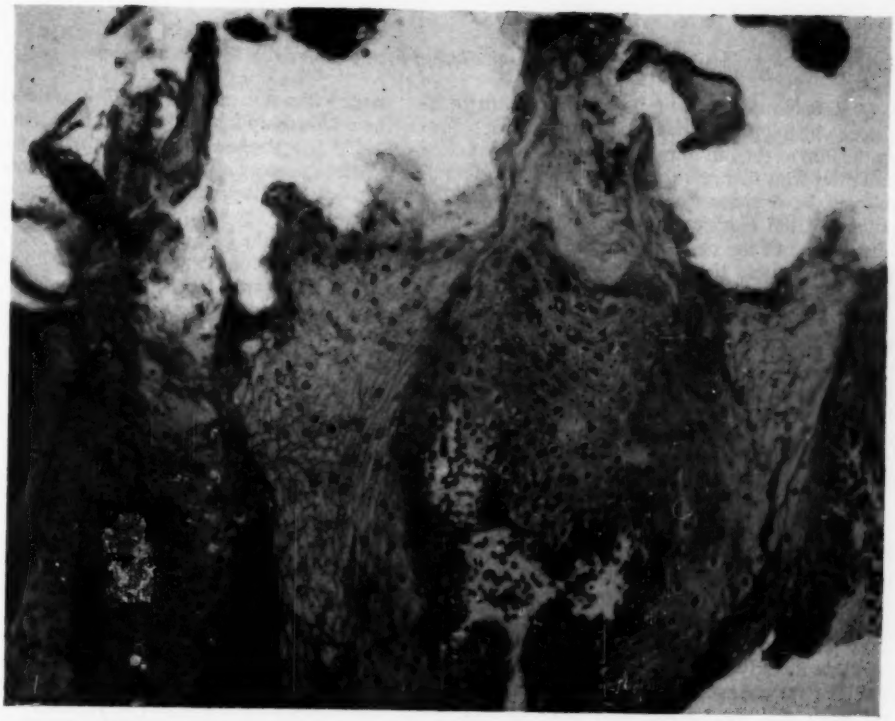

FIG. 4

FIG 5

Oesophagus We have failed to observe any consistent histological change in the oesophageal epithelium as seen in biopsies of our patients before and after iron therapy. Counts of cell layers and of mitoses have shown no significant changes. The nature of the biopsies does not of course permit observation of the oesophageal muscle coats. Fragments of muscle tissue were, however, present in some biopsies and appeared histologically normal.

The only abnormal epithelial pattern was seen in Case 11 (second biopsy), which showed patchy areas of abnormal maturation, the superficial layers instead of being flattened, retained the columnar form of the basal layer so that the surface of the mucosa was very irregular. The third biopsy of this case, however, showed a normal pattern. There was no dysphagia in this case.

DISCUSSION

The smooth appearance of the tongue seen in some cases of iron-deficiency anaemia is due to the disappearance of the filiform papillae. When iron therapy is given, new papillae are evident clinically after one to two weeks, and biopsy after one month's therapy has confirmed their reappearance. The lingual changes are a sensitive index of iron repletion and the tongue returns to normal long before angular stomatitis and koilonychia have disappeared. The tongue biopsies reveal that there is no evidence of increased regenerative activity in the basal-cell layers of the epithelium following iron therapy. There was considerable variation in the numbers of filiform papillae per high-power field in the iron-deficiency cases before therapy. However, the mean number of filiform papillae was more than doubled after the iron store had become replete. Our findings show a correlation between filiform papillae and iron repletion.

The return to normal is marked by the appearance of foci of keratohyalin granules in the Malpighian cells and the formation of keratinous papillae at these sites. Keratohyalin granules are known to be constantly associated with keratin formation, though whether they are concerned in its synthesis or represent a degeneration product, is uncertain. They are rich in calcium mucopolysaccharides and alkaline phosphatase (Lorincz and Stoughton, 1958) but contain no detectable iron. Keratin itself contains large amounts of cystine. Chemical analysis of nail clippings in cases of koilonychia associated with hookworm infestation (Jalili and Al-Kassab, 1959) has shown that the cystine content of the nail keratin is reduced in this condition. It may be that there is a relationship between body iron levels and the metabolism of sulphur-containing amino-acids. Both koilonychia and glossal atrophy may, however, occur without overt evidence of iron deficiency, and the relationship is clearly not a simple one. Beutler (1957) has suggested that the symptoms of iron deficiency may be related to disturbances of tissue metabolism rather than simply to a lowered haemoglobin level. The body iron stores may be depleted even though the blood haemoglobin is normal.

Beutler believes that in patients with latent iron-deficiency anaemia there may be a deficiency of other iron-containing respiratory intermediates. In rats depleted of iron by repeated bleeding he found that the amount of cytochrome C in the liver and kidneys fell before there was a drop in the haemoglobin. This work casts doubt on the claims of Hahn and Whipple (1936) that the iron-containing intracellular respiratory pigments are maintained inviolate in iron-deficiency states at the expense of haemoglobin. Low serum cyanocobalamin (vitamin B_{12}) levels have recently been reported in iron-deficiency anaemia (Cox, Meynell, Gaddie, and Cooke, 1959). At present it can only be speculated that the link between iron metabolism and keratin synthesis may be found in the iron-containing pigments concerned with intracellular respiration. Present-day histochemical techniques are not sufficiently advanced to permit estimation of the content of respiratory pigments and enzymes of individual cells. Jacobs (1960) found three patients with iron-deficiency anaemia who showed keratinization and parakeratosis of the buccal mucosa.

In our present series of lingual biopsies there appears to be a close correlation between histological appearances and the clinical description of atrophy. Although some tongues which appear clinically to be normal showed only a few filiform papillae, none of the atrophic tongues had more than one filiform papilla per high-power field.

The Paterson-Kelly syndrome is now a rarity in Great Britain. Only two cases were found in over 18 months' search among cases of iron-deficiency anaemia (Cases 13 and 14). The biopsy findings in the present cases did not confirm the hyperkeratinization and atrophic change reported by Suzman (1933) and Savilahti (1946) at necropsy. There was no evidence of any epithelial lesion in the oesophagus in our patients (with or without dysphagia). Neural or muscular abnormality could not, however, be excluded, since the biopsies only reached the submucosa in most cases.

Two patients complained of dysphagia which disappeared after treatment. No abnormality in the oesophageal epithelium was found in any of the patients either before or after therapy. This finding has recently been confirmed by Jacobs (1960) who noted a patient with postcricoid carcinoma arising from a normal epithelium. It is clear that changes in

the oesophageal epithelium do not accompany glossal atrophy and the relationship of post-cricoid carcinoma to possible oesophageal hyperkeratinization in iron-deficiency anaemia is doubtful.

We would like to thank Dr. D. E. Price, consultant pathologist, Barnsley Hospitals for cooperation. Great help and encouragement throughout this work is acknowledged to Professor D. H. Collins, Department of Pathology, University of Sheffield, and to the staff of this department.

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Carbohydrates and sulphur-containing compounds in the anaemic buccal epithelium

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SYNOPSIS Mucosal biopsies from the inside of the cheek show epithelial atrophy, glycogen depletion, and an occasional increase in sulphydryl content in both iron-deficiency and megaloblastic anaemias. These changes are the same in the two types of anaemia but are more marked in the latter group.

Atrophy of the squamous epithelium of the mouth may occur in both iron-deficiency anaemia and anaemia due to vitamin B_{12} deficiency (Jacobs, 1960). The changes are more common and more marked in the latter. They are not related to the degree of anaemia. The occasional finding of hyperkeratotic and parakeratotic lesions in these anaemic mucosae has prompted the investigation of carbohydrates, sulphydryl and disulphide groups, all of which are known to be concerned with the process of keratinization (Rothman, 1954).

MATERIAL

Suction biopsy specimens were obtained from the buccal mucosa by the method previously described (Jacobs, 1960). The following groups of patients were examined.

1 FORTY-FOUR PATIENTS WITH IRON-DEFICIENCY ANAEMIA All had a haemoglobin concentration of less than 9 g. per 100 ml. In only one was the serum iron concentration greater than 60 μg. per 100 ml. and in 21 it was less than 20 μg. per 100 ml.

2 EIGHTEEN PATIENTS WITH MEGALOBLASTIC ANAEMIA DUE TO VITAMIN B_{12} DEFICIENCY These comprised 16 cases of pernicious anaemia and two cases of multiple jejunal diverticula. Serum B_{12} levels were estimated in 13 of the cases and only in two patients with pernicious anaemia did the level exceed 60 $\mu\mu$g. per ml. In these, values of 100 and 135 $\mu\mu$g. per ml. respectively were obtained.

3 THIRTY-NINE NON-ANAEMIC PATIENTS CONVALESCING FROM OTHER DISORDERS Their ages varied from 19 to 83 years.

METHODS

All specimens were fixed in 10% formol saline. They were embedded in paraffin, and 5μ sections were cut vertically through the tissue and stained with haematoxylin and eosin. Periodic-acid-Schiff (P.A.S.) was used to visualize carbohydrate substances (Pearse, 1960). Control sections were treated with salivary diastase at 37°C. for 30 minutes and some with diastase followed by hyaluronidase (Hyalase) at 37°C. for 24 hours. Thionin and toluidine blue were both used to investigate metachromasia (Pearse, 1960). Barrnett and Seligman's dihydroxy-dinaphthyl-disulphide (D.D.D.) method as modified by Bahr (1957) was used for the demonstration of sulphydryl groups and its specificity was shown by pre-treatment of control sections with N-ethyl maleimide (Pearse, 1960). Sections treated with thioglycollic acid before incubation with D.D.D. were used to demonstrate total sulphydryl and disulphide concentration (Pearse, 1960).

Serum iron was estimated by the method of Ramsay (1957), and serum vitamin B_{12} was kindly estimated by Dr. I. Chanarin using *Lactobacillus leishmannii*. Marrow aspirates were fixed in 10% formol saline and sections stained for haemosiderin using Perls's method (Pearse, 1960).

RESULTS

EPITHELIAL THICKNESS The maximum epithelial thickness in each specimen is shown in Fig. 1. In the normal group no specimen was found which had a thickness of less than 300 μ. In the iron-deficiency group a quarter (11) had a maximum thickness of less than 300 μ and in the group of megaloblastic anaemias over half (10) of the specimens were thinner than normal. There was no relationship between epithelial thickness and haemoglobin concentration, serum iron level, serum vitamin B_{12} concentration, or the amount of stainable iron in the bone marrow in either the iron-deficient or the vitamin-B_{12}-deficient patients. Five of the iron-deficient group showed koilonychia and all these had a buccal epithelial thickness of 250μ or less.

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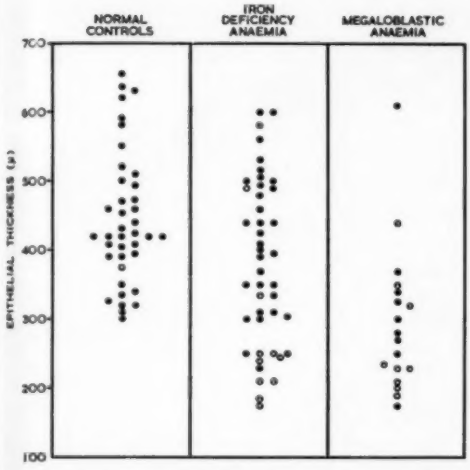

FIG. 1. *Maximum epithelial thickness in buccal biopsies from anaemic and non-anaemic patients.*
● *Normal glycogen content*
○ *Scanty or absent glycogen*

GLYCOGEN Glycogen is demonstrated as a P.A.S.-positive substance which is removed by digestion with salivary diastase. The normal buccal epithelium contains abundant glycogen (Fig. 2). It is not present in the basal layer but appears in the prickle cell layer and its maximum concentration is just below the surface of the epithelium or sometimes in the surface cells themselves. In some cases the most superficial squamous cells contain no glycogen at all. In one of the non-anaemic group of patients only a trace of glycogen was found in the mucosa.

In 44 cases of iron-deficiency anaemia there were 10 where the epithelium contained only a trace of glycogen or no glycogen at all. Among the 18 cases of megaloblastic anaemia nine showed an absence of glycogen or only trace amounts in the epithelium. In both anaemic groups there is a tendency for the most atrophic epithelium to be deficient in glycogen (Fig. 1). This diminution of glycogen is not apparently related to the level of haemoglobin, serum iron, or serum vitamin B_{12}, or to the amount of bone marrow haemosiderin.

NON-GLYCOGEN POLYSACCHARIDES In the normal epithelium a homogeneous substance giving a positive P.A.S. reaction and resisting salivary digestion is found in the intercellular spaces. It is particularly prominent and dense in the superficial layers where the intercellular prickles have disappeared and the intercellular space is reduced.

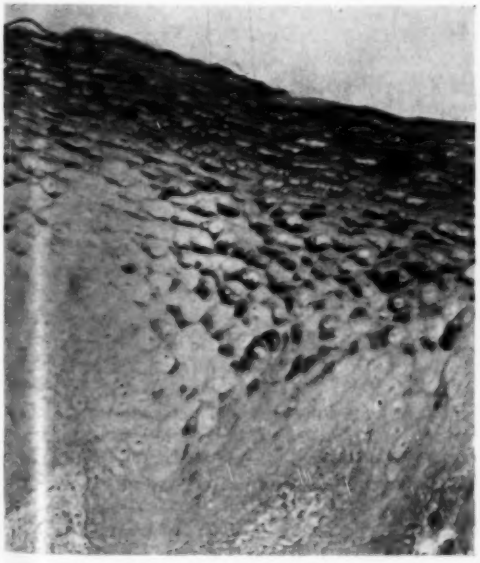

FIG. 2. *Normal buccal mucosa. Glycogen present in the superficial part of the epithelium. P.A.S.,* × *150.*

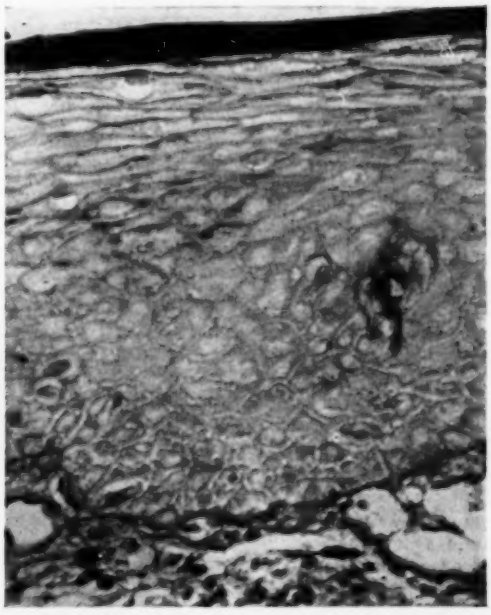

FIG. 3. *Atrophic mucosa from a case of iron-deficiency anaemia. Abnormal polysaccharide present in the most superficial cells. P.A.S. following diastase,* × *300.*

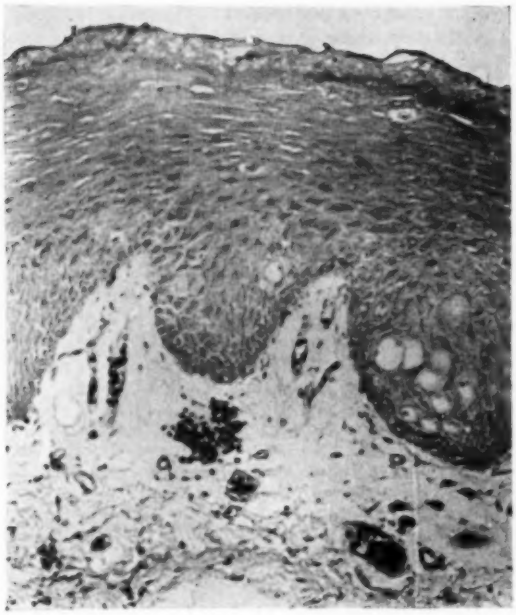

FIG. 4

FIG. 5

FIG. 4. *Normal buccal mucosa. D.D.D., × 150.*

FIG. 5. *Atrophic mucosa from a case of iron-deficiency anaemia. There is intense staining in the superficial layer indicating an increase in sulphydryl concentration. D.D.D., × 300.*

Staining of the basement membrane by this method varies in intensity between specimens. These polysaccharide substances do not differ in the anaemic and the non-anaemic groups. Very occasional cells in the most superficial layer of the epithelium contain a cytoplasmic fleck of a P.A.S.-positive substance resisting salivary digestion. Only in one normal specimen was more than a trace of this intracellular carbohydrate found. It was present in significant, though still small, amounts in the superficial cells of six cases of iron-deficiency and in four cases of megaloblastic anaemia (Fig. 3). Its presence was not specifically related to the presence or absence of glycogen or to the thickness of the epithelium. It was also unrelated to the degree of anaemia, iron, or vitamin B_{12} deficiency. In the specimens where this polysaccharide substance was present digestion with hyaluronidase at 37°C. for 24 hours did not remove it, neither did it show metachromatic staining with thionin or toluidine blue.

SULPHYDRYL AND DISULPHIDE GROUPS All the normal biopsy specimens gave a positive reaction for sulphydryl groups using the D.D.D. method. This was present throughout the full thickness of the epithelium. Staining was considerably reduced by prior incubation with N-ethyl maleimide. In many of the specimens the reaction was weak but in 18 cases there was some increase of staining in the superficial layer (Fig. 4). Six cases showed a slight increase in the amount of staining after treatment with thioglycollic acid.

The appearance in the anaemic groups was similar to the normal except in four cases of iron-deficiency and in three cases of vitamin B_{12} deficiency which showed an increase in staining, particularly in the superficial layer (Fig. 5). This was entirely due to an increased concentration of sulphydryl groups, as prior incubation with thioglycollic acid did not intensify the staining. The increase of sulphydryl concentration was invariably associated with an abnormal amount of the intracellular polysaccharide in the superficial cells noted above.

VITAMIN B_{12} In addition to the estimations carried out in cases of megaloblastic anaemia, serum levels

were determined in 30 patients with iron-deficiency anaemia, from some of whom biopsies were not taken. All were within the normal range (150 to 850 $\mu\mu$g. per ml.) and showed no relation to the level of haemoglobin, serum iron, or bone marrow haemosiderin.

DISCUSSION

There have been few studies of the histology of the buccal mucosa and the present findings must necessarily be viewed in the light of observations made on the other squamous epithelial surfaces of the body. Much is known of the epithelium comprising the epidermis and the vaginal lining, though neither of these two sites has precisely the same physiology and pathology as the buccal mucosa. Even within the oral cavity there is probably some variation in the metabolism of the epithelium from one site to another.

Many observations have been made on the normal and abnormal gingiva and the results are of interest. It seems generally agreed that the normal gingival epithelium may contain glycogen (Trott, 1957; Turesky, Glickman, and Fisher, 1959; Weiss, Weinmann, and Meyer, 1959) although Dewar (1955) states that this is only present as a response to inflammation. It is found in about 30% of cases and Turesky *et al.* (1959) note that it is usually found in those epithelia which have deep rete pegs. Most of these authors agreed that it is increased in incidence and amount in the presence of gingivitis but Berg, Lundquist, Schram, and Fosdick (1947) found a diminution in inflamed gingiva. There is no variation with age, sex, or menstrual phase and the amount present is not related to the amount of glycogen in the vaginal epithelium (Trott, 1957).

The epithelium covering the alveolar process is probably more akin to the buccal epithelium obtained in the present investigation than the frequently inflamed and traumatized gingival margin. Weiss *et al.* (1959), comparing the two sites, state that keratinization, which occurs in up to 30% of gingival specimens, never occurs in the alveolar mucosa. The glycogen of the alveolar squamous cells is also much higher than that found at the gingival margin. In both these respects the picture is similar to that found in the present series of normal buccal epithelia. The alveolar glycogen is apparently unaffected by the presence of inflammation.

Normal skin contains little stainable glycogen (Rothman, 1954) while in the vagina glycogen appears to be inversely related to the degree of keratinization and directly related to the epithelial thickness (Ayre, 1951).

The present series of biopsies confirms the previous observation of epithelial atrophy in some cases of anaemia (Jacobs, 1960) and shows that whereas the normal buccal mucosa usually contains abundant glycogen this is often lacking in anaemic subjects. The glycogen deficiency is usually associated with thinning of the epithelium. It has already been noted that in the gingiva it is the thinner epithelia which tend to be devoid of glycogen. Similarly in the vagina epithelial atrophy following cessation of oestrogen stimulation is associated with a disappearance of glycogen. Although Cruickshank and Sharman (1934) believed that the presence of glycogen in the vagina was the direct result of oestrogen stimulation, Ayre (1951) thought that the presence or absence of glycogen was a direct consequence of the thickness of the epithelium. The accumulation of glycogen in squamous cells some distance from their blood supply, by virtue of their distance from the basement membrane, may be an indication of energy production by an anaerobic mechanism (Ayre, 1951). This view is not entirely supported by the present finding of occasional cases of glycogen depletion occurring in buccal epithelia of normal thickness. Weisberger and Fischer (1960), studying leukoplakia of the cheek, have also noted glycogen depletion in mucosa some distance from the lesions and as yet morphologically normal.

The present results of staining by the D.D.D. method appear to reveal a rather lower concentration of sulphydryl groups in the buccal mucosa than that found in the gingiva by Turesky, Crowley, and Glickman (1957). This may be explained by the occurrence of keratinization in the gingiva. An increase in sulphydryl content was found by these workers in the presence of gingivitis. The increased sulphydryl content in a few of the anaemic mucosae examined may indicate a tendency towards keratinization. These cases always showed, in addition, the presence of an intracellular P.A.S.-positive diastase-stable substance in the superficial squamous cells. It is possible that this material may be similar to that found by Steiner (1958) in some parakeratotic lesions of skin. The P.A.S.-positive staining of squamous cell nuclei seen by Steiner was not found in the present series.

The histological findings of epithelial atrophy, glycogen depletion, and a possible tendency to keratinization or parakeratosis in the buccal mucosa appear to be the same in the two types of anaemia investigated. No specific findings characteristic of one group alone were discovered. The only striking difference between the two groups was the occurrence of koilonychia in some of those iron-deficient patients with the severest degree of epithelial atrophy in the mouth. This probably never occurs in megaloblastic anaemias.

Cox, Meynell, Gaddie, and Cooke (1959)

demonstrated vitamin B_{12} deficiency arising as a consequence of iron-deficiency anaemia. This suggests the possibility that the mucosal changes may all be a result of B_{12} deficiency. The greater prominence of these changes in the megaloblastic group lends support to this possibility. Dabski (1960), basing his conclusions on the observation of exfoliated cells from the mouth, believes this mechanism to operate. Low serum B_{12} levels in iron-deficiency anaemia comparable to those found by Cox *et al.* (1959) have not been found in the present limited series of cases. Furthermore there is no relation between histological changes and serum vitamin B_{12} levels in either the iron-deficient or megaloblastic cases in this series. The apparently greater effect of the megaloblastic anaemias on the buccal mucosa might be explained by reference to the long period of subclinical B_{12} deficiency which probably precedes all overt clinical signs of pernicious anaemia.

It seems likely that the basic metabolic disorders in the epithelium in iron and vitamin B_{12} deficiency are different. The present investigation, however, has demonstrated only some common manifestations and throws no light on the primary defects.

I should like to thank Professor D. M. Pryce and Mr. I. R. H. Kramer for their encouragement, Miss P. Evans for her technical assistance, and the Medical Research Council for a grant supporting this work.

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J. clin. Path. (1961), **14**, 615

Hereditary elliptocytic anaemia

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SYNOPSIS A sibship with four cases of hereditary elliptocytic anaemia is described. The condition in this family may have arisen as a mutation in the mother of the sibship; affected members were unable to taste phenylthiocarbamide while normal members were tasters.

Experiments with ^{32}P-orthophosphate *in vitro* did not show any evidence of biochemical upset as found in hereditary spherocytosis; thus a combination of congenital spherocytosis and elliptocytosis cannot be supported as the cause of the haemolytic state. Clinical evidence of haemolytic disease was accompanied by a tendency to excessive lysis *in vitro*.

Infection may play a part in the precipitation of anaemic crises in this as in other hereditary haemolytic anaemias.

Elliptical erythrocytes occur as a well recognized hereditary anomaly in man. The first record of elliptical human red corpuscles is usually credited to Dresbach (1904) but Lambrecht (1938) states that the anomaly was observed in 1860 by Goltz. Its inheritance as a regular autosomal dominant has been confirmed in many studies since its nature was first established as familial by Bishop (1914) and as hereditary by Hunter and Adams (1929). Now it is known that in some families at least the elliptocytic trait shows genetic linkage with the rhesus blood groups (Goodall, Hendry, Lawler, and Stephen, 1953; Marshall, Bird, Bailey, and Beckner, 1954; Morton, 1956; Clarke, Donohoe, Finn, McConnell, Sheppard and Nicol, 1960).

Hereditary elliptocytosis is not usually associated with anaemia. Thus, until 1943, only 12% of recorded cases showed a significant anaemia of haemolytic type (Penfold and Lipscomb, 1943). This hereditary anomaly has to be differentiated from the elliptocytosis occurring in other types of anaemia, notably pernicious anaemia, hypochromic microcytic anaemia, and the anaemias of myelosclerosis and lymphoid neoplasms.

The purpose of this paper is to report a six-year study of the clinical course and haematological investigations of a sibship of seven, four of whom have elliptocytosis with a significant haemolytic anaemia and splenomegaly. One member required cholecystectomy for pigment stones; another derived benefit from splenectomy.

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CLINICAL DETAILS

The propositus, patient II 3 (Fig. 1), a 49-year-old farm-worker was admitted to hospital with a five-day history of upper abdominal pain, nausea, vomiting, and slight jaundice. One similar attack two years previously had resolved with conservative treatment. Examination showed epigastric tenderness, slight enlargement of the liver, a palpable tender spleen, and mild conjunctival icterus. His urine contained a trace of bile and excess urobilinogen. Radiological examination of the chest, barium meal examination, and cholecystography revealed

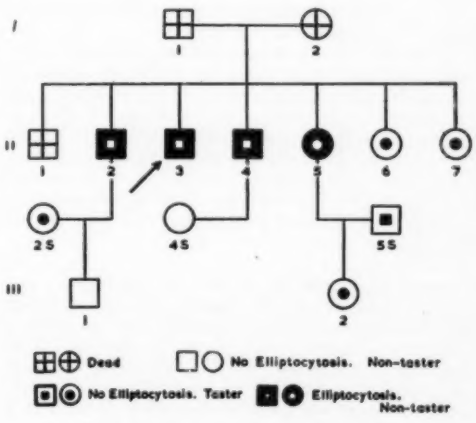


FIG. 1. *The pedigree of the affected family.*

TABLE I

HAEMATOLOGICAL FINDINGS IN THE SIBSHIP

Patient and Date of Samples		*Hb (g./100 ml.) 100% = 14·8 g.)*	*R.B.C. (ml./c.mm.)*	*P.C.V. (%)*	*M.C.H.C. (%)*	*M.C.V. (cμ)*	*Reticulocytes (%)*	*W.B.C. (cells/c.mm.)*	*Direct Coombs Test*	*Alkaline Phosphatase (K.-A. units)*	*Serum Bilirubin (mg./100 ml.)*		
											Indirect	*Direct*	*Total*
II 2	30/ 5/57	14·08	4·06	37	38	91	7·2	—	Neg.	14	—	—	—
	18/10/59	13·6	4·2	36	37	86	6·4	—	Neg.	12	0·9	0·4	1·3
II 3	6/ 6/56	13·12	4·2	37	35	88	5·6	—	Neg.	8	1·5	0·5	2·0
	22/ 5/60	12·32	4·1	35	35	85	9·2	—	Neg.	9	2·0	0·8	2·8
II 4	*Pre-splenectomy*												
	25/5/59	9·04	3·8	—	—	—	1	2,800	—	—	—	—	—
	28/5/59	10·4	3·9	34	38	87	4·3	2,800	Neg.	8	1·0	0·4	1·4
	13/7/59	10·88	3·63	33	33	90	6·1	3,200	Neg.	9	1·4	0·6	2·0
	Splenectomy	12/8/59	(1 pint blood transfused)										
	Post-splenectomy												
	20/ 8/59	13·44	4·4	39	34·4	—	1	4,000	—	—	—	—	—
	10/12/59	15·68	5·06	43	36	85	1	6,100	Neg.	9	0·8	0·32	1·14
	5/ 7/60	14·56	5·1	42	34	82	0·9	4,800	Neg.	8·5	0·6	0·44	1·02
II 5	24/9/59	12·16	3·44	32	37	94	9·2	—	Neg.	9	1·4	0·6	2·0
	29/2/60	11·52	3·86	34	34	88	7	—	Neg.	6	1·3	0·4	1·7
II 6	24/10/54	13·7	4·25	37	37	87	0·5	—	Neg.	15	—	—	—
	18/10/59	14·56	4·82	41	35	85	0·7	—	Neg.	12	—	—	0·3
II 7	24/10/55	15·04	4·8	43	35	89	0·6	—	Neg.	9	—	—	—
	18/10/60	14·4	4·65	40	36	86	0·8	—	Neg.	9	—	—	0·2

no abnormality. There was a mild normochromic anaemia with 90% elliptocytes, occasional microelliptocytes and spherocytes, and a reticulocytosis of 7%. Haemolytic elliptocytic anaemia was diagnosed in this patient and his siblings (Tables I to III). Tests of hepatic function showed raised serum bilirubin, thymol turbidity, and cephalin-flocculation values. The patient responded to conservative treatment but on discharge three weeks later the thymol turbidity and cephalin-flocculation levels were still rather high and it was concluded that slight, chronic hepatic damage was present. This was later verified by biopsy. No other sibling has similar hepatic damage.

In January 1957 he suffered a further haemolytic crisis preceded by an attack of bronchitis.

In February 1957 a posterior gastro-jejunostomy was performed for a large posterior duodenal ulcer. At operation it was noted that the gall bladder was normal and contained no calculi, and that the spleen was approximately three times normal size. Liver biopsy showed chronic inflammatory thickening of the capsule, a fibrous scar with round cell infiltration, and a focus of bile-duct hyperplasia. After gastro-jejunostomy his dyspepsia was relieved and he has had no further haemolytic episodes.

PATIENT II 2 This 56-year-old brother had a haemolytic episode at the age of 34. He was seen by his family doctor who diagnosed 'pernicious anaemia', but he made a spontaneous recovery without specific treatment. Five years later, he had biliary colic for which cholecystectomy was performed at another hospital. The gall bladder contained numerous small pigment stones. He has a compensated haemolytic anaemia (Table I) with slight conjunctival icterus and a palpable spleen.

PATIENT II 4 This 48-year-old brother appears to have the most labile haematological state of the affected members as he has suffered from recurrent nausea, vomiting, weakness, anaemia, and jaundice since childhood.

In May 1959 he was admitted to hospital with pyrexia, epigastric pain, nausea, vomiting, and a cough productive of mucopurulent sputum (Table I). Repeated sputum culture revealed normal flora. Crepitations were heard at the base of the right lung and radiological examination showed slight opacification at the right costophrenic angle. There was no hepatomegaly but the spleen was enlarged and tender. His urine contained no bile, but urobilinogen was detected at a 1 : 25 dilution by Ehrlich's test. He responded to a course of antibiotics but recurrent crises and anaemia with resultant incapacity for work seemed to warrant splenectomy. After investigation this was performed in August 1959 during a phase of remission (Mr. G. M. Sturrock). At operation the gall bladder appeared normal; in particular it contained no calculi. Liver biopsy revealed no histological abnormality. Marrow biopsy showed a hyperplastic, normoblastic reaction. The normoblasts were round, as were the majority of the polychromatic corpuscles.

The enlarged spleen (500 g.) was uniformly congested but showed no focal lesion. Imprints from freshly cut surfaces showed only occasional erythrophages. Histological examination confirmed the congestion and showed hyperplasia of the reticuloendothelial cells of the red pulp with diffuse haemosiderosis.

Since discharge in September 1959 the patient has returned to full work. His haematological state has remained stable and satisfactory. Details and comparison of studies performed before and after splenectomy are shown in tables.

PATIENT II 5 This 47-year-old sister has a life-long history of anaemia, but does not admit to having been jaundiced. At one time she was treated elsewhere with folic acid for a macrocytic anaemia not associated with a histamine-fast achlorhydria. Since coming under observation she has had a fairly well compensated haemolytic state with no crises.

PATIENTS II 6 AND II 7 These sisters of the propositus are symptom-free and have normal blood. They have very kindly acted as normal controls in various investigations.

PATIENT II 1 This brother, who died at the age of 19 from 'spinal trouble', had no history of anaemia or jaundice.

GENETIC STUDIES

Both parents of the affected sibship are dead (Fig. 1), but it is known that the mother had anaemia, gallstones, and intermittent jaundice.

In order to investigate the possibility of elliptocytosis in collaterals, all living paternal and maternal siblings, *i.e.*, brothers and sisters of I 1 and I 2, all their living offspring, and all living offspring of deceased siblings were examined by one of us (R.J.L.D.). Screen tests, made on finger-prick samples, including Hb estimation (as oxyhaemoglobin in an EEL photoelectric colorimeter), reticulocyte count (Brecher's method), and examination of Leishman-stained films. In preparing the films care was taken to spread them lightly to give a relatively thick smear, as the elliptical shape may be lost by thin, hard spreading. Any member with an abnormal screen test or history of anaemia was more fully investigated, the further investigations including tests of osmotic fragility and autohaemolysis. In all, 27 paternal and 29 maternal relatives were examined. None was shown to have significant anaemia or any familial anomaly of the erythrocytes. As the maternal history is compatible with a haemolytic anaemia and such a condition is present only in her offspring, we think it is likely that this familial elliptocytosis arose as a mutation in the mother of the affected sibship.

A link between the elliptocytosis and the rhesus blood groups was sought, but because of the similarity of the Rh genotype of the affected and normal members it could not be established. Data on the ABO, MN, and P systems were similarly uninformative. On the other hand, a study of their ability to taste phenylthiocarbamide (P.T.C.) showed the four affected members to be non-tasters and the normal members to be tasters. The numbers, however, are so small that linkage could not be established. Among other elliptocytic families, families 3 and 4 of Goodall *et al.* (1953 and 1954) and family 5 of Lawler and Sandler (1954) have been similarly tested (Lawler, personal communication). The data were such that no conclusions could be reached in families 3 and 4, but there appeared to be independence of tasting and elliptocytosis in family 5. The findings in this family suggest that future studies of families with hereditary elliptocytosis should include ability to taste phenylthiocarbamide, particularly if there is no evidence of Rh linkage or if there is a consistently associated haemolytic anaemia.

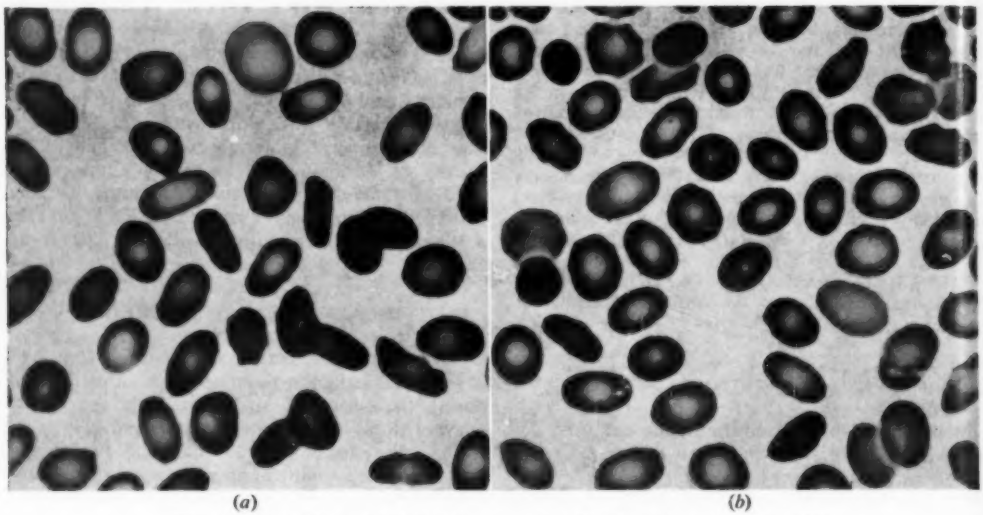
HAEMATOLOGICAL INVESTIGATIONS

MORPHOLOGY All members of the sibship have had at least four complete haematological investigations during the period of study, but only the results relevant to the subsequent text are given below.

The affected members all showed a similar red cell morphology with approximately 90% elliptocytes, occasional microelliptocytes, spherocytes, and cells which tended to crenate even in fresh samples. A variable reticulocytosis was always present. After splenectomy in patient II 4 (Fig. 2) the number of microelliptocytes and spherocytes was significantly increased, as has been noted in several previously reported cases (Dacie, Mollison, Richardson, Selwyn, and Shapiro, 1953; Lipton, 1955; Letman, 1955; Wilson and Long, 1955).

The presence of microelliptocytes and spherocytes in the peripheral blood, the splenomegaly, and the haemolytic anaemia in all the affected members suggested the possible coexistence of congenital spherocytosis and elliptocytosis, and the following investigations were undertaken to see if this combination really existed.

RADIOCHEMICAL INVESTIGATION *in vitro* The rate of exchange of ^{32}P-orthophosphate between plasma and erythrocytes in cases of congenital spherocytosis has been found to be normal (Prankerd, Altman, and Young, 1954, 1955) but it was later found by Tabechian, Altman, and Young (1956) that in the presence of sodium fluoride spherocytes behaved differently from normal red cells. Thus when NaF (10^{-3}M) is added to normal blood there is little or no alteration in the rate of ^{32}P-orthophosphate uptake by the erythrocytes, but with congenitally spherocytic cells the exchange process ceases after about one hour and may even be reversed. Two cases of congenital spherocytosis were examined by us and showed the fluoride inhibition but blood from the four siblings with elliptocytosis as well as from their unaffected sisters behaved normally. Patient II 4 gave similar results before and after



(*a*) (*b*)

FIG. 2. *Blood films* (*a*) *before splenectomy and* (*b*) *after splenectomy* × 1,200.

splenectomy. Thus, from the results of this radiochemical test, we have no reason to believe that either the spherocytosis or the anaemia in our elliptocytic cases was due to a coexistent congenital spherocytic anaemia. Three other cases of familial elliptocytosis with no anaemia and six normal controls likewise showed no inhibition of exchange on addition of NaF. Studies on the ^{32}P-orthophosphate exchange in hereditary elliptocytosis have not been previously reported and details of these findings are to be published elsewhere.

OTHER CHEMICAL INVESTIGATIONS Haemoglobin from the affected siblings showed a normal electrophoretic behaviour and virtually no alkali resistance. Liver function tests (including quantitative estimation of plasma proteins by both precipitation and electrophoresis) were normal except in patient II 3, who showed increased cephalin-flocculation and thymol turbidity levels.

FRAGILITY AND AUTOHAEMOLYSIS TESTS Saline osmotic fragility (fresh and incubated) and autohaemolysis tests were carried out according to Dacie (1960a), except that heparinized blood was used for all tests. The normal values quoted are derived from studies by one of us (R.J.L.D.) on 34 normal men and 30 normal women.

The splenic venous sample from patient II 4 was obtained at the time of operation, after the splenic artery had been ligated. The Hb level on this sample was 11·5 g. per 100 ml.; haematocrit 38%. The results of these tests are recorded in Tables II and III. The total bilirubin content of the splenic venous sample was 3·2 mg. per 100 ml., whereas

TABLE II

OSMOTIC FRAGILITY

Patient		*Median Cell Fragility (% NaCl.)*	
		Fresh Blood	*After Sterile Incubation for 24 Hours*
II 2		0·448	0·576
II 3		0·432	0·616
II 4	Pre-splenectomy	0·44	0·60
	Splenic venous blood	0·48	0·72
	Post-splenectomy	0·466	0·66
II 5		0·46	0·568
II 6		0·42	0·54
II 7		0·4	0·548
Normal range		0·384 to 0·464	0·492 to 0·60

TABLE III

AUTOHAEMOLYSIS

Patient		*% Haemolysis*			
		24 Hours		*48 Hours*	
		No Glucose Added	*Glucose Added*	*No Glucose Added*	*Glucose Added*
II 2		1·2	0·3	9·5	1·2
II 3		0·8	0·4	5·0	1·3
II 4	Pre-splenectomy	0·6	0·3	6·0	1·2
	Post-splenectomy	1·3	0·8	17·0	4·1
II 5		1·0	0·5	10·0	2·3
II 6		0	0	0·9	0·3
II 7		0	0	1·5	0·2
Normal range		0·0-0·5	0·0-0·3	0·5-5·0	0·1-0·5

that of a peripheral venous sample was 1·1 mg. per 100 ml.; this marked difference suggests that in this patient the spleen was exerting an excessive haemolytic action on the abnormal erythrocytes.

DISCUSSION

THE ELLIPTICAL SHAPE In hereditary elliptocytosis the factors determining the unusual shape of the erythrocytes are unknown. As in other such cases, Hb electrophoresis and alkali denaturation in this family revealed no abnormal haemoglobins. It has been shown, however, by Breuer, De Vries, Peket, and Matoth (1958) that special moving-boundary electrophoretic techniques reveal minor differences between normal adult haemoglobin and haemoglobins from patients with elliptocytosis and other hereditary anaemias; these observers postulate a weakness of the bonds that link the subunits of the haemoglobin molecule and thus the internal structure of these abnormal erythrocytes. The only report of significantly high levels of foetal haemoglobin in adult elliptocytic cases is that of Ducla-Soares and Parreira (1958). A structural or enzymic defect of the stroma or cell membrane is a possible but yet unproven explanation of the elliptical shape, and in our cases with haemolytic anaemia at least, such a defect seems probable in view of the abnormal haemolysis *in vitro*.

OSMOTIC FRAGILITY *in vitro* The osmotic fragility values of all the affected members in this family were significantly higher than those of the unaffected members and were at or above maximum normal levels. This supports the view that the presence of bizarre-shaped cells, microelliptocytes, and spherocytes is likely to be associated with hyperhaemolysis.

After splenectomy, in patient II 4 there was a significant increase in the osmotic fragility of both fresh and incubated samples, explicable no doubt by the increase of microelliptocytes and spherocytes in the peripheral blood. An increase in fragility in elliptocytosis is generally accepted as occurring after splenectomy, but in only a few cases has a comparison been made with pre-splenectomy values in the same patient, and in these cases the changes varied from a marked increase (Letman, 1955) to no increase at all (Blackburn, Jordan, Lytle, Swan, and Tudhope, 1958). Similarly, in congenital spherocytosis, Young, Izzo, and Platzer (1951) and Dacie (1960b) conclude that there is a further increase in osmotic fragility following splenectomy, particularly as measured on incubated samples.

Study of venous blood from the splenic vein itself has been thus far reported only once in relation to elliptocytosis, no significant differences being noted in the red cell morphology or osmotic fragility range in samples from the splenic vein and artery (Lipton, 1955). In contrast, we found a considerable increase in the osmotic fragility of splenic venous blood in patient II 4 compared with that of peripheral venous samples, and since this could not be explained by any difference in the packed cell volume of the samples it may reflect a splenic action on the cells promoting lysis in a way comparable with that seen in congenital spherocytosis (Emerson, Shen, Ham, Fleming, and Castle, 1956).

AUTOHAEMOLYSIS Reports on autohaemolysis in hereditary elliptocytosis are few and the results inconclusive. In this family the results are similar to those obtained by Dacie (1960c) in his families C, D, and G, namely, that increased levels were found on sterile incubation for both 24 and 48 hours and the addition of glucose greatly reduced the haemolysis but not to normal levels.

After splenectomy our patient II 4 showed a further increase of autohaemolysis. The case D.Hi of family B described by Dacie (1960d) also showed an abnormally high level but had not been studied before splenectomy. The rise in our case and the high level in Dacie's case contrast with the situation in congenital spherocytosis where post-splenectomy autohaemolysis levels tend to be lower than those recorded before splenectomy (Selwyn and Dacie, 1954).

THE HAEMOLYTIC ANAEMIA Most cases of hereditary elliptocytosis have neither clinical nor laboratory evidence of haemolytic anaemia. A minority, however, show various degrees of hyperhaemolysis but the factors determining these grades of severity are only partly understood. The homozygous state, which is so important in the haemoglobinopathies, has only twice been found responsible for severe haemolytic, elliptocytic anaemia (Wyandt, Bancroft, and Winship, 1941; Lipton, 1955).

The presence of spherocytes in the blood of heterozygous subjects with elliptocytic anaemia has led observers in the past to suggest the possibility of a combination of congenital spherocytosis and elliptocytosis as the basis for the anaemia but neither Holst-Larsen (1947) nor subsequent workers have offered any proof of this idea, and certainly the results of our genetic and radiochemical studies are against such a combination. A possible, and, to us, a reasonable explanation of the presence of the microspherocytes and microelliptocytes is that these small forms are the result of red cell fragmentation (Motulsky, Singer, Crosby, and Smith, 1954).

The rare combination of the elliptocytic trait with

a haemoglobinopathy is not of any help in the elucidation of the usual type of hereditary elliptocytic anaemia, and, in the known examples of combination with Hb S (Vandepitte and Louis, 1955) and Hb C (Avery, 1956), the abnormal haemoglobin did not appear to potentiate the pathogenicity of the elliptocytosis.

It has been suggested that anaemia may be caused by the combination of the gene(s) for elliptocytosis with another gene which of itself does not cause any detectable change in the blood. Such a possibility was raised by the cases of Mason (1938) and Lendval (1949), where there was a history of an undetermined type of anaemia on the side of the parent not bearing the elliptocytic trait. In sum, there is no convincing proof that the anaemia of hereditary elliptocytosis is due to combination with other genetically determined abnormalities.

INFECTION AND HAEMOLYSIS OR HAEMOPOIETIC DEPRESSION In the congenital haemolytic anaemias it is still uncertain whether the relationship of a casual infection to a clinical exacerbation of the haematological state is an intensification of the haemolysis or a suppression of haemopoiesis, although the observations of Owren (1948), Dameshek and Bloom (1948), and Gasser (1950, 1951) all point to temporary marrow failure as the more important precipitating factor.

All the affected members in our elliptocytic family gave a history of episodes of fever, weakness, and anaemia associated with abdominal pain or respiratory symptoms. Patient II 4 on his first admission to hospital had such an episode with a productive cough but culture of the sputum yielded no pathogenic organisms; the finding of leucopenia in the presence of a mild respiratory infection, anaemia, and a reticulocyte count well below his 'compensated' level certainly suggested that the anaemia was due to a sudden haemopoietic insufficiency. Unfortunately, confirmatory marrow aspiration was not carried out. Campanacci, Torlontano, Tonietti, and Conti (1960) suggested that their two elliptocytic anaemic patients had hypoplastic erythropoiesis, and although no other comparable reports are available, infection seems to have unmasked the presence of previously unsuspected hereditary elliptocytosis by precipitating a severe anaemia in Dacie's (1954) case and that reported by Blackburn *et al.* (1958).

MEGALOBLASTIC CHANGES The reputedly megaloblastic relapse in Cases II 2 and II 5 were diagnosed elsewhere but not documented precisely enough to be cited as indubitable. We have seen two other cases of elliptocytic anaemia mistakenly diagnosed on the blood film as megaloblastic anaemias. Megaloblastic change is known to occur rarely in cases of congenital spherocytosis and other haemolytic anaemias, particularly in pregnancy, but no adequately proven case has been recorded in a person with hereditary elliptocytosis.

HAEMOLYTIC ROLE OF THE SPLEEN The spleen is usually enlarged and palpable in patients with elliptocytic haemolytic anaemia, but in those patients with merely the trait the spleen is not palpable, and, therefore, probably not enlarged. The haemolytic activity of the spleen has been demonstrated in patient II 4 by bilirubin estimation and osmotic fragility tests on splenic venous blood. As in congenital spherocytosis, the cause of the splenic congestion and the mechanisms leading to destruction of the abnormal erythrocytes are largely unexplained. The available evidence suggests, however, that the lytic role of the spleen is secondary to the genetically determined defect of the erythrocytes.

TREATMENT Although the factors responsible for the elliptical shape of the erthrocytes and the pathogenesis of the occasionally associated haemolytic anaemia are far from being adequately understood, there seems little doubt that in the anaemic cases splenectomy alleviates the haemolytic state and improves the physical well-being of the patient. The results of splenectomy are reviewed by Dacie (1960e) and we merely add that in our Case II 4 the operation was of definite benefit.

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The plasma clearance of injected doses of folic acid as an index of folic acid deficiency

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SYNOPSIS The folic acid clearance test of Chanarin (1958) has been studied in normal white subjects, patients with megaloblastic anaemia, healthy Bantu males, Bantu females, hospital patients without anaemia, and in patients with scurvy.

The test clearly distinguishes patients with megaloblastic anaemia, where clearance from the plasma of injected folic acid is abnormally rapid, from normal subjects. The rate of clearance of folic acid is abnormally rapid both in vitamin B_{12} and in folic acid deficiency. Rapid clearance of injected folic acid in patients with primary B_{12} deficiency may be the result of a conditioned deficiency of folic acid.

The test is sufficiently sensitive to detect body deficiency of folic acid before the development of anaemia.

In patients with scurvy, folic acid is cleared from the plasma abnormally rapidly. It is suggested that this may be the result of an associated dietary deficiency of folic acid.

While the assay of vitamin B_{12} in serum provides a sensitive and specific index of body deficiency of that vitamin, there is no generally accepted parallel procedure for the diagnosis of folic acid deficiency. The recognition that deficiency of vitamin B_{12} may precede the development of anaemia by a considerable period (Callender and Denborough, 1957) makes the development of a sensitive test for body deficiency of folic acid necessary.

Attempts have been made to develop specific and sensitive procedures for the diagnosis of folic acid deficiency. Spray, Fourman, and Witts (1951) injected small doses of folic acid and measured the amount excreted in the urine. Patients with folic acid deficiency excreted less of the injected dose than did control subjects. The test is of limited sensitivity and the small volume of urine assayed greatly multiplies any technical errors.

Assay of folic acid in fasting serum with *L. casei* has been claimed by Herbert, Baker, Frank, Pasher, Sobotka, and Wasserman (1960) to delineate megaloblastic anaemias due to primary folic acid deficiency from those with vitamin B_{12} deficiency, but Cooperman, Luhby, and Avery (1960) found the overlap among normal and folic acid deficiency so great that the determination was of no value as an index of folic acid deficiency.

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Formiminoglutamic acid (FIGLU), an intermediate in the metabolism of histidine, accumulates in the urine in subjects with folic acid deficiency (Broquist, 1956; Luhby, 1957; Silverman, Gardiner, and Condit, 1958; Broquist and Luhby, 1959; Spray and Witts, 1959). However, there appears to be some doubt as to the sensitivity of the methods for its detection. Davidson and Jandl (1959) state that the presence of FIGLU in the urine does not appear to be an entirely satisfactory test for folic acid deficiency at present, and Herbert *et al.* (1960) do not consider the method dependable for detecting folic acid deficiency.

Chanarin, Mollin, and Anderson (1958a) injected intravenously a standard dose of folic acid and measured its rate of clearance from the plasma. In all patients with megaloblastic anaemia due to folic acid deficiency and in nine of 20 patients with megaloblastic anaemia due to vitamin B_{12} deficiency, the clearance of the injected folic acid was more rapid than in control subjects. Chanarin, MacGibbon, O'Sullivan, and Mollin (1959) subsequently studied 250 non-anaemic pregnant females. 11 patients with normal twin pregnancy, and 11 patients with untreated megaloblastic anaemia of pregnancy. The plasma clearance of folic acid was abnormally rapid in two-thirds of pregnant subjects in late pregnancy, and in all patients with twin pregnancy.

The rate of clearance was most rapid in patients with megaloblastic anaemia of pregnancy. Although the test did not distinguish between deficiency of vitamin B_{12} and folic acid, it was invariably abnormal in megaloblastic anaemias due to folic acid deficiency, and was sufficiently sensitive to detect body deficiency of folic acid before the development of anaemia.

In the South African Bantu, megaloblastic anaemias are commonly encountered, particularly in association with pregnancy, and in malnourished infants (Metz, 1960). In these patients, serum vitamin B_{12} levels may be subnormal but are not usually as low as in patients with pernicious anaemia. Folic acid therapy invariably induces haematological remission, while the results of pharmacological doses of vitamin B_{12} are uncertain (Metz, 1960). With a high incidence of malnutrition in this ethnic group, there is possibly a great deal of subclinical folic acid deficiency. The folic acid clearance test of Chanarin appears to be a promising index of folic acid deficiency; this paper presents then the results of the application of this test in groups of normal subjects, Bantu and white patients with megaloblastic anaemia, and Bantu subjects not suffering from anaemia.

METHODS

The folic acid clearance test was carried out as described by Chanarin *et al.* (1958a). A dose of 15 μg. folic acid per kg. body weight was injected intravenously, and blood drawn at three, 15, and 30 minutes later. The serum was separated and stored at −20°C. until assayed. Serum folic acid was assayed microbiologically with *Str. faecalis* as described by Jukes (1955) and Chanarin, Anderson, and Mollin (1958b). Standard sera were included in each assay. Each specimen was assayed in triplicate in at least two, and usually in three, separate batches.

Serum vitamin B_{12} was assayed with *L. leishmanii* as described previously (Brandt and Metz, 1961). Normal values range from 160 to 1,300 $\mu\mu$g./ml.

SUBJECTS STUDIED

NORMAL WHITES Thirteen female and 19 male White laboratory staff or medical students who were not anaemic.

MEGALOBLASTIC ANAEMIA ASSOCIATED WITH PREGNANCY Twenty-one patients with megaloblastic anaemia, comprising 19 Bantu presenting after delivery, and two Whites in the last trimester of pregnancy.

PERNICIOUS ANAEMIA Twelve Whites and three Bantu patients with megaloblastic anaemia and subnormal serum vitamin B_{12} levels. Vitamin B_{12} by intramuscular injection induced complete haematological remission in all.

SCURVY Three Bantu adults with the classical clinical features of scurvy.

BANTU MALES Eight members of staff not suffering from anaemia.

BANTU FEMALES Eight hospital patients who were not anaemic convalescing from diseases not usually affecting folic acid metabolism.

RESULTS

NORMAL WHITES The differences in the mean folic acid levels in male and female subjects are not statistically significant (Table I). The mean levels at three, 15, and 30 minutes after the intravenous injection of folic acid are slightly higher than those reported by Chanarin *et al.* (1958a) but the ranges at 15 and 30 minutes in the two studies are similar. The lower limit at three minutes is similar in the two series but in the present study the upper limit at three minutes is higher than that of Chanarin *et al.* (1958a). We confirm the observations of Chanarin that after the intravenous injection of a dose of 15 μg. folic acid per kg. body weight, serum folic acid levels of less than 75 μmg./ml. at three minutes, 26 μmg./ml. at 15 minutes, and 4 μmg./ml. at 30 minutes are abnormal.

TABLE I

SERUM FOLIC ACID IN NORMAL MALES AND FEMALES

No.	*Males Folic Acid (μmg./ml.)*			*Females Folic Acid (μmg./ml.)*		
	3 Min.	*15 Min.*	*30 Min.*	*3 Min.*	*15 Min.*	*30 Min.*
1	87	27	8	96	52	14
2	91	52	25	122	22	12
3	94	35	19	128	58	24
4	110	20	20	144	48	13
5	118	43	9	148	45	16
6	128	91	12	154	54	22
7	130	27	20	158	46	15
8	134	53	35	160	60	24
9	138	38	19	163	54	29
10	140	45	38	164	42	16
11	142	75	21	168	62	36
12	148	55	28	173	61	33
13	154	50	17	180	62	43
14	170	49	39			
15	174	25	11			
16	178	29	9			
17	182	60	25			
18	190	46	24			
19	219	73	24			
20	220	65	27			
21	260	49	30			
Mean	152·7	47·9	21·9	150·6	51·2	22·8
S.D.	44	17	8·8	22	11	9·6

MEGALOBLASTIC ANAEMIA ASSOCIATED WITH PREGNANCY In 19 patients, the rate of clearance of injected folic acid was abnormally rapid; in the other two patients, the three- and 15- minute levels were within normal limits, while the 30-minute level of 4 μmg./ml. was at the lower limit of normal

TABLE II

SERUM FOLIC ACID AND SERUM VITAMIN B_{12} LEVELS IN PATIENTS WITH MEGALOBLASTIC ANAEMIA ASSOCIATED WITH PREGNANCY

No.	Folic Acid (μmg./ml.)			Vitamin B_{12} (μμg./ml.)
	3 Min.	15 Min.	30 Min.	
1	10	0	0	650
2	10	1	0	440
3	12	2	0	Contaminated
4	21	4	3	430
5	34	1	0	100
6	37	0	0	200
7	39	0	0	370
8	41	0	0	275
9	41	0	0	970
10	44	0	0	1,155
11	46	8	9	130
12	49	8	3	200
13	60	8	3	270
14	65	4	2	150
15	73	2	0	830
16	89	14	6	580
17	113	26	4	230
18	124	2	2	10
19	126	29	4	575
20	156	13	3	Contaminated
21	250	16	11	250
Mean	68	6·5	2·4	411

(Table II). In the 19 patients with abnormal clearance patterns, the three-minute level was subnormal in 15, the 15-minute level subnormal in all, and the 30-minute level subnormal in 16. The results are similar to those of Chanarin *et al.* (1958a) who found rapid clearance of injected folic acid in 11 patients with megaloblastic anaemia of pregnancy.

PERNICIOUS ANAEMIA Fourteen patients showed an abnormally rapid clearance of injected folic acid, while in one patient the three- and 15-minute levels were within normal limits with the 30-minute level at the lower limit of normal (Table III). Of the 14 patients with abnormal patterns, the three-minute level was subnormal in 13, the 15-minute level subnormal in 13, and the 30-minute level subnormal in 13.

TABLE III

SERUM FOLIC ACID AND SERUM VITAMIN B_{12} LEVELS IN PATIENTS WITH PERNICIOUS ANAEMIA

No.	Folic Acid (μmg./ml.)			Vitamin B_{12} (μμg./ml.)
	3 Min.	15 Min.	30 Min.	
1	13	0	0	80
2	26	10	2	10
3	28	0	0	50
4	30	0	0	30
5	35	0	0	10
6	38	0	0	25
7	42	0	0	160
8	42	2	2	100
9	46	18	2	150
10	48	10	2	150
11	50	3	2	115
12	55	48	4	60
13	66	13	0	10
14	83	3	0	65
15	99	23	4	52
Mean	47	8·6	1·2	71

SCURVY The three subjects with scurvy showed abnormally rapid clearance of injected folic acid from the plasma. The three-, 15-, and 30-minute levels were 30, 10, 0 μmg./ml., 47, 0, 0 μmg./ml., and 45, 4, 1 μmg./ml. respectively.

BANTU MALES In all but two of the healthy Bantu males studied, the serum folic acid levels were above the lower limit of normal. In these two subjects, one showed a subnormal three-minute level, and in one the 15-minute value was subnormal (Table IV).

TABLE IV

SERUM FOLIC ACID AND SERUM VITAMIN B_{12} LEVELS IN HEALTHY BANTU MALES AND IN NON-ANAEMIC HOSPITALIZED BANTU FEMALES

No.	Males Folic Acid (μmg./ml.)			Females Folic Acid (μmg./ml.)		
	3 Min.	15 Min.	30 Min.	3 Min.	15 Min.	30 Min.
1	64	24	12	17	7	0
2	80	27	14	78	13	3
3	95	31	17	103	20	0
4	115	30	18	104	25	13
5	195	38	19	125	21	11
6	225	32	19	168	38	24
7	240	11	9	190	19	1
8	250	61	44	248	53	4
Mean	153	31	19	129	27	9

BANTU FEMALES In four of the group of eight non-anaemic Bantu females the clearance pattern was abnormal (Table IV). In one subject the three-, 15-, and 30-minute folic acid levels were all subnormal and three others showed subnormal 30-minute levels.

DISCUSSION

The serum folic acid levels after intravenous injection of folic acid in the normal subjects studied are similar to those reported by Chanarin *et al.* (1958a). There is a clear difference in the folic acid levels in normal subjects and in patients with megaloblastic anaemia, the 15- and 30-minute levels providing the most significant differences. The folic acid clearance test will therefore establish deficiency of folic acid where the deficiency is severe enough to produce megaloblastic anaemia.

The rapid clearance of injected folic acid in a significant number of Bantu females not suffering from anaemia confirms that the test is able to establish folic acid deficiency before the development

of anaemia, and suggests that subclinical folic acid deficiency is not uncommon in this group. In the Bantu males studied, the clearance pattern is more similar to that of normal Whites, and correlates with the observation that megaloblastic anaemia due to primary folic acid deficiency is rarely seen in this group (Metz, 1960).

Rapid clearance of injected doses of folic acid in patients with scurvy possibly indicates that in human scurvy there is an associated dietary deficiency of folic acid, resulting in depletion of body folic acid stores. It has been shown that folic acid deficiency with megaloblastic anaemia regularly develops in scorbutic monkeys fed on a milk diet (May, Hamilton, and Stewart, 1952).

The finding of rapid clearance of injected doses of folic acid in severely anaemic patients with primary viatmin B_{12} deficiency (pernicious anaemia) confirms the observations of Chanarin *et al.* (1958a). The folic acid clearance test is thus unable to distinguish megaloblastic anaemia due to primary B_{12} deficiency from that due to primary folic acid deficiency. These findings can be explained on the basis that deficiency of vitamin B_{12} may condition body deficiency of folic acid, the rate of clearance of injected folic acid in patients with primary vitamin B_{12} deficiency being then an index of the degree of depletion of folic acid in these patients. Secondary folic acid deficiency in patients with primary B_{12} deficiency would also explain the finding of FIGLU in the urine in B_{12} deficiency (unpublished observations quoted by Herbert *et al.*, 1960) and suggests that any test of folic acid deficiency would be unable to distinguish between primary folic acid deficiency and that conditioned by vitamin B_{12} deficiency. Secondary folic acid deficiency can be demonstrated in experimentally produced vitamin B_{12} deficiency in sheep (Dawbarn, Hine, and Smith, 1958). That the converse may apply, *i.e.*, vitamin B_{12} deficiency conditioned by primary folic acid deficiency, is suggested by the subnormal levels of serum vitamin B_{12} in some patients with megaloblastic anaemia associated with pregnancy. In these patients, however, vitamin B_{12} deficiency may be occurring *pari passu* with that of folic acid.

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Platelets in blood stored in untreated and siliconed glass bottles and plastic bags

I Studies *in vitro*

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SYNOPSIS The number and function (clot retraction) of platelets in blood stored at 4°C. for three weeks in plastic bags and in untreated and siliconed glass containers were determined using EDTA and acid-citrate dextrose (ACD) as anticoagulants. No essential differences were found.

During recent years platelet transfusions have been used with increasing frequency in the treatment of thrombocytopenic bleeding, but there are still many unsolved problems connected with this treatment which are of both practical and theoretical importance. The present paper is mainly concerned with the containers and anticoagulants used for collecting blood as Cronkite and Jackson (1959) have recently given an excellent survey of the broader aspects of platelet transfusion.

It is generally claimed that platelets are preserved and handled much better in siliconed glass or plastic containers than in untreated glassware. As a matter of fact, it has been claimed that ACD-blood in untreated glass bottles (conventional blood-bank bottles) is without any value as a platelet source, for example, in the treatment of patients with thrombocytopenia (Gardner, 1958; Stefanini and Dameshek, 1955; Tullis, 1953). However, certain investigations have recently shown that the importance of using siliconed equipment in handling platelets seems to have been overemphasized (Lundevall, 1958; Hellem, 1960). McIlvanie (1958) compared plastic, untreated, and siliconed glass containers for platelet transfusions, and he found that untreated glass containers gave results which were only slightly inferior to those obtained with plastic and siliconed glass. This is in accordance with my own experience from many years' work with blood platelets, including experience in the treatment of thrombocytopenia with concentrated thrombocyte suspensions. I have found that platelets from blood collected in ordinary untreated glass containers are quite valuable, even after storage for about 24 hours.

It is of importance for clinicians, and, especially, for blood bank staffs, to know how to handle blood in order to secure a high recovery of viable platelets when the blood is transfused, and it is also of financial importance. The following questions illustrate some aspects of the problem: Is it necessary to use fresh blood? If not, how long is it safe to store the blood before use? Is it necessary to use siliconed glass bottles? Are plastic bags superior to glass bottles? A study of the literature does not give a satisfactory answer to these questions.

Plastic bags are a modern and very elegant blood-transfusion equipment but the relative importance of the advantages and disadvantages has not yet been solved (Dudley, Richmond, McNair, Paton, and Cumming, 1958; Gibson, 1958; Kissmeyer-Nielsen, 1960). It is certain, however, that plastic equipment, at least for the present, is expensive in comparison with the conventional glass equipment. This paper attempts to show essential quantitative and qualitative differences in platelets stored in various containers. Our results, however, did not give any definite answer, and consequently these studies were followed by investigations *in vivo* in which the survival of platelets stored in various containers was determined. The results are published in Part II.

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MATERIAL AND METHODS

PLATELET COUNTS It was necessary to choose a reliable method which could be used for counting platelets in blood stored for three weeks, taking into consideration that spontaneous sedimentation of erythrocytes in plasma disappears quickly when blood is stored.

For counting platelets, the contents of the container were thoroughly mixed, and 1 ml. of blood was transferred to an untreated test tube containing 4 ml. of 3·8% sodium citrate ($2H_2O$). The contents of the tube were mixed, and the tube was left at room temperature for about 15 minutes for sedimentation of the red cells. From the middle of the supernatant a small volume was transferred to a Thoma counting chamber (height 0·05 mm.), which was left for sedimentation of the platelets in a moist chamber for at least 15 minutes. The platelets in 40 small squares were counted, and this figure multiplied by 1,000 gives the number of platelets in 1 $mm.^3$ of whole blood (ignoring the volume of the red cells in the mixture of 1 ml. whole blood + 4 ml. citrate). The accuracy of the method was calculated from 25 duplicate determinations on blood bottles immediately after bleeding. The difference between the two values varied between 0 and 20,000 (S.D. 6,400, and coefficient of variation 2·7%). The number of platelets in the 25 bottles immediately after bleeding, without correction for the volume of anticoagulant added, varied between 159,000 and 360,000 (mean 255,000). The platelet counts were always performed in duplicate by the same technician.

PLATELET FUNCTION It is difficult to assess the function, *i.e.*, the viability, of platelets *in vitro*, but this is supposed to be of major interest, especially in view of the experiments of Hjort, Perman, and Cronkite (1959) who showed that the viability of the platelets was of essential importance for their haemostatic function in dogs.

We made an attempt to assess the viability by determining the ability of the stored, washed platelets to give clot retraction of platelet-free, fresh, citrated plasma. The platelets from the stored blood were isolated by differential centrifugation. Platelet-rich plasma was produced by centrifugation at about 300 *g* for five minutes. (The centrifugal force is calculated for the middle of the test tube.) The platelet-rich plasma was transferred to siliconed test tubes; 1/10 by volume of 2% triton (WR 1339) in 0·9% saline was added, and the mixture was centrifuged at about 600 *g* for 30 minutes. The supernatant was discarded, and the platelets were then washed twice in saline with 1/10 by volume of triton 2%. After the last washing the platelets were resuspended in 0·9% saline without triton. The amount of saline added was adjusted by platelet counts, so as to give a platelet suspension containing about 10^6 platelets per mm^3. The ability of this platelet suspension to give clot retraction was assessed in the following mixtures:—

One millilitre platelet-free, normal citrate plasma (the platelet-free plasma was prepared from 1 ml. 3·8% citrate + 9 ml. blood centrifuged at 1,300 *g* for 30 minutes); 0·2 ml. of the washed platelet suspension; and 0·1 ml. 0·25 M $CaCl_2$.

The test was performed in round-bottomed test tubes (75 by 10/11 mm.) pre-heated in a Bunsen flame almost to melting point. This pre-heating is, as shown by Lüscher (1956), essential, as it completely abolishes any adhesion between the clot and the glass. After clotting the tubes were left undisturbed at 37°C. for one hour. After this time the serum formed was carefully removed using a fine Pasteur pipette, taking care not to squeeze the clot. The volume of serum was measured, and this volume as a percentage of the total volume (1·3 ml.) represents the clot retraction. Using this method for 32 determinations in 16 normal subjects, values between 85 and 97% were found (mean 92, S.D. 2·9). The accuracy of the method was calculated from 12 duplicate determinations on normal subjects. The standard deviation was found to be 1·9 and the coefficient of variation 2·1%. As in the platelet counts, the clot retraction was determined in duplicate.

It was hoped that it would also have been possible to investigate platelet function during storage using the above-mentioned washed platelet suspension in the classical thromboplastin-generation test (Biggs and Douglas, 1953), mixing the platelet suspension with normal $Al(OH)_3$-adsorbed plasma and normal serum. However, the platelet function remained absolutely unaltered during the three weeks of storage; consequently, the results of this part of the investigation are not again referred to.

BLOOD CONTAINERS, ANTICOAGULANTS, AND DONORS Unselected donors and unselected blood containers were used for the experiments. All the bleedings

TABLE I

CONTAINERS AND ANTICOAGULANTS USED

Container	*Number*	*Anticoagulant*	*Amount of Blood (ml.)*
Untreated glass	5	ACD (Danish Pharmacopoea) 150 ml. Citric acid, H_2O 4·8 g., Na_3-citrate, $2H_2O$ 13·3 g., dextrose, H_2O 30 g. Distilled water to 1,000 ml.	500
Siliconed glass	5	ACD (Danish Pharmacopoea) 150 ml.	500
Plastic (Fenwal)	5	ACD (Unites States Pharmacopoea) 75 ml. Formula A	500
Untreated glass	5	EDTA 50 ml. 1·5% Na_2EDTA, $2H_2O$ and 0·7% NaCl	500
Siliconed glass	5	EDTA 50 ml.	500
Plastic (Fenwal)	5	EDTA 50 ml.	500

were performed by the head nurse of the blood bank or by myself. The various containers and anticoagulants used are shown in Table I.

CLEANING OF GLASSWARE AND BOTTLES The cleaning procedure used may be of some importance in the experiments under consideration. The glassware used was of our routine pattern and no special precautions were taken. Test tubes were treated as follows:—

Immediately after use they were immersed in a detergent, which is a 0·5 solution of equal parts of technical Na_2CO_3, sodium metasilicate, and trisodium phosphate. The test tubes were boiled for 10 to 15 minutes in this solution, rinsed three times in running tap water, immersed for at least half an hour in 1% HCl, rinsed three times in running tap water, immersed in technical demineralized water, and finally rinsed once in running demineralized water and dried in a hot oven at 80°C.

The bottles were cleaned in a semi-automatic washing machine at the hospital pharmacy. The cleaning procedure included 2% NaOH + 1% trisodium phosphate, 80°C., 2% trisodium phosphate, 60°C., tap water, 60°C., cold tap water, decalcified tap water, and drying in a hot oven.

COATING OF GLASSWARE WITH SILICONE The bottles were siliconed in the hospital pharmacy, after cleaning with sulphuric chromic acid, using silicone M.S. 1107 dissolved in neutral, water-free trichloroethylene followed by rinsing in distilled water. The bottles were baked at 180°C. for three hours. The test tubes were siliconed, using a 10% solution of silicone M 441 (I.C.I.) in petroleum ether, followed by rinsing in several changes of distilled water and air-drying (no baking).

RESULTS

In a preliminary experiment, the number and function of blood platelets in five untreated and five siliconed glass bottles were followed for three weeks.

All the bottles contained ACD, 150 ml. and 500 ml. of blood. (ACD, according to the Danish Pharmacopoea, is composed of citric acid 4·8 g., Na_3 citrate, 2 H_2O 13·3 g., dextrose 30 g. in 1,000 ml. distilled water.)

Containers were examined immediately after the bleeding, and after three, seven, 14, and 21 days of

TABLE II

PLATELETS AND ABILITY FOR CLOT RETRACTION AFTER STORAGE IN VARIOUS CONTAINERS

Time of storage in days		*0*		*3*		*7*		*14*		*21*		*Total Percentage Decrease in Platelet Count*
Untreated glass	ACD	283	81·5[1]	276	89·8	211	64·8	187	24·6	122	23·7	54
	EDTA	248	88·8	244	94·4	237	86·3	169	72·7	118	49·2	52
Siliconed glass	ACD	199	93·6	226	89·0	207	66·0	178	33·1	144	29·2	36
	EDTA	279	93·5	266	91·3	210	87·9	186	63·1	155	35·5	44
Plastic (Fenwal)	ACD	229	91·2	213	81·2	144	47·8	99	51·8	87	25·3	62
	EDTA	239	91·6	252	89·1	182	83·9	146	80·7	118	27·6	51

[1]The first figure in each column is the number of platelets (× 1,000), and the second is the clot retraction. Each figure is the average of the results obtained for the five containers included in each group.

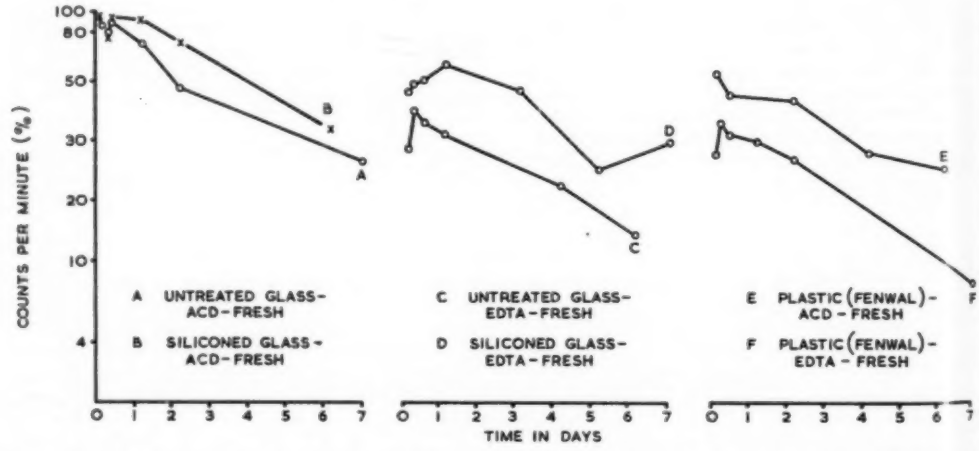
COUNTS PER MINUTE (%)
100
80
50
30
20
10
4
A UNTREATED GLASS - ACD - FRESH
B SILICONED GLASS - ACD - FRESH
C UNTREATED GLASS - EDTA - FRESH
D SILICONED GLASS - EDTA - FRESH
E PLASTIC (FENWAL) - ACD - FRESH
F PLASTIC (FENWAL) - EDTA - FRESH
0 1 2 3 4 5 6 7
TIME IN DAYS

FIG. 1. *Number and function of platelets stored in untreated and siliconed glass and plastic bags when ACD was used as anticoagulant.*

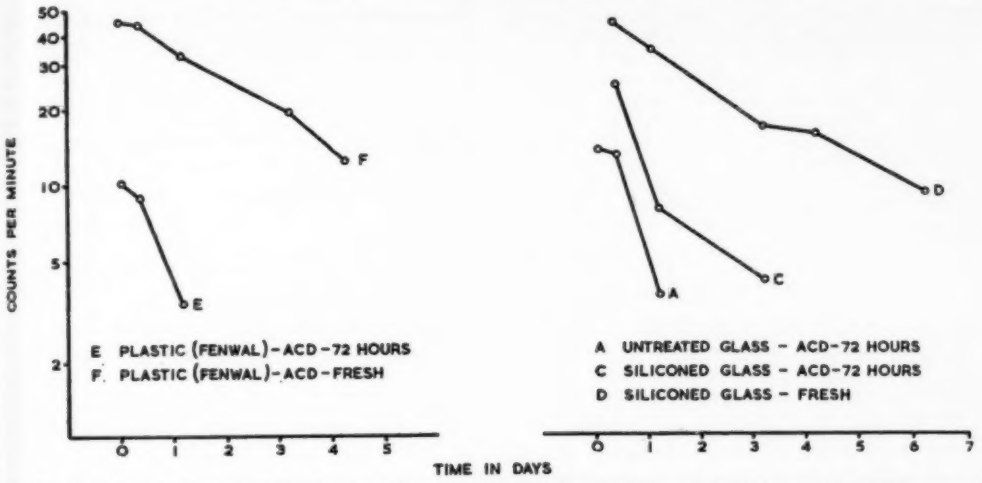

FIG. 2. *Number and function of platelets stored in untreated and siliconed glass and plastic bags when EDTA was used as anticoagulant.*

storage at 4°C. Contrary to expectation, it was found that the number of platelets and their clot-retraction ability decreased identically in the two groups of containers. During the three weeks the average number of platelets in both groups decreased by 61% of the original value.

Immediately after bleeding, isolated platelets from both groups gave a clot retraction of 90%; after three weeks the results were 28 and 27% respectively.

The number of platelets and the clot retraction remained unaltered during the first three days of storage.

The investigations were repeated on six more representative groups of containers and anticoagulants (Table I). Each group contained five specimens, and consequently the whole material included 30 'pints' of blood.

The EDTA solution used was the Fenwal formula, although it was realized that this solution, because of lack of dextrose, was supposed to give haemolysis during storage.

The number and function of the platelets were investigated immediately after the withdrawal of the blood and after three, seven, 14, and 21 days of storage at 4°C.

The results are set out in Table II and Figs. 1 and 2.

DISCUSSION

The results obtained with the different containers and the two different anticoagulants were remarkably similar. The only times during storage at which any differences were noticed were after seven and 14 days. At these times, the EDTA-stabilized blood showed a less marked decrease in clot retraction than the ACD-stablized specimens. However, this difference was not apparent until after one week, and it was not uniform.

These studies *in vitro* do not allow any conclusions as to the superiority or inferiority of any of the three containers and the two anticoagulants included in the investigation. The results are thus of limited value, except for the fact that they give the impression that the claimed superiority of siliconed glass and plastic equipment in relation to number and viability of platelets may be wrong. However, the studies performed were only *in vitro*, and determinations of platelet survival might give quite a different result.

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II Survival studies

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SYNOPSIS Platelet survival was determined using untreated and siliconed glass bottles and plastic bags (Fenwal) for collecting and storing blood. The platelets were tagged *in vivo* with P^{32} in six polycythaemic patients undergoing treatment with P^{32}. The results showed that fresh ACD blood collected in untreated glass, siliconed glass, and plastic gave the same recovery of platelets in the recipients. The use of EDTA (Fenwal formula) as anticoagulant gave results inferior to those obtained with blood using ACD as anticoagulant. Even after storage up to 24 hours in untreated glass bottles (ordinary bank blood) a satisfactory recovery of platelets was observed. After storage for 72 hours the recovery was less but not negligible.

We have tried to solve part of the problem of preserving platelets for subsequent transfusion and for use in extracorporeal circulation by determining the survival of platelets in blood delivered in different containers, using ACD and EDTA as anticoagulant and storing the blood for periods of varying length before transfusion. It is difficult to determine the survival of thrombocytes but it is possible by using platelets tagged with various isotopes. A survey of the methods used was recently published by Aas and Gardner (1958). Tagging is usually performed *in vitro*, and the rather rough treatment to which the platelets are exposed during tagging has some influence on the results obtained, consequently labelling *in vitro* is not ideal for an investigation in which a relatively small difference in survival time might indicate superiority or inferiority of one container compared with another. We therefore used platelets tagged *in vivo* with P^{32}. We used blood from patients with polycythaemia who were treated with P^{32}. The technique employed is very similar to that used by Adelson, Rheingold, and Crosby (1957) in dogs and human beings. It is well known that tagging is maximal in about a week after the administration of the drug (Adelson *et al.*, 1957). Using blood from patients with polycythaemia gives another advantage, namely, the possibility of drawing several pints of blood from the same donor at very short intervals. The various 'pints' drawn from the same donor contain the 'same' platelets marked in exactly the same way *in vivo*, thus affording an ideal opportunity for comparing the effect of storage on platelets in various containers before transfusion.

MATERIALS AND METHODS

DONORS Patients with polycythaemia undergoing treatment with P^{32} were used as donors. They were given about 5 μ. P^{32} by mouth and after an interval of five to seven days bleedings were started, 500 ml. of blood being drawn on each occasion, and the bleedings were continued daily for three to six days, depending on the clinical and haematological condition of the patients. Six patients were included in the study, and delivered a total of 24 'pints' of blood. Two patients delivered six bottles each, two four bottles each, one three bottles, and the last donor only one. All the bleedings were performed either by the head nurse of the blood bank or by one of the authors (F. K-N). Automatic shaking was used during bleeding into glass bottles.

RECIPIENTS It was felt that it was undesirable and unnecessary to use 'normal' recipients, so recipients were patients from the Department of Radiotherapy undergoing heavy *x*-ray therapy for advanced malignant tumours, mostly carcinoma of the cervix uteri. The patients were all in a good clinical condition, but some of them had previously been transfused several times (Table I). The transfusions were preceded by screening for irregular blood-group antibodies (papainized cells and indirect Coombs technique) and by cross-matching

including the use of the indirect Coombs test. Most of the patients were investigated for platelet antibodies using Dausset's technique for complete (Dausset and Malinvaud, 1954) and Coombs consumption technique for incomplete antibodies. The Coombs consumption technique used was a modification of the technique elaborated by Moulinier (1955) and Steffen (1955). All the transfusions were given by one of the authors (F. K-N).

BLOOD CONTAINERS, ANTICOAGULANTS, AND STORAGE BEFORE DETERMINATION OF PLATELET SURVIVAL Untreated and siliconed glass bottles and plastic bags (Fenwal) were included in the study. In the majority of the experiments ACD (Danish or United States Pharmacopoea, formula A) was used, but in three experiments EDTA (Fenwal formula) was employed. Most of the transfusions were given as fresh blood as quickly as possible after the bleeding, but in some experiments the platelet survival was determined after storage at 4°C. for periods varying between 15 and 72 hours. The exact composition of the various anticoagulants used is given in Part I.

DETERMINATION OF PLATELET SURVIVAL The principle of the method was determination of the activity of all isolated, washed platelets from an exact volume of plasma at different times after the transfusion. The method was a modification of the technique used by Aas and Gardner (1958). Exactly 19 ml. of blood was drawn into an untreated test tube containing 1 ml. of EDTA 5%. The contents were mixed; the haematocrit value (Wintrobe) was determined, and exactly 4·5 ml. of blood was transferred to four untreated test tubes (100 × 15/16 mm.) containing a mixture of 1 ml. EDTA 5% and 3 ml. dextran (Pharmacia) (M_w = 153·000) in 0·9% saline. The contents of the tubes were thoroughly mixed and centrifuged at 175 *g* for 15 minutes. Exactly 20 ml. of the supernatant was transferred to siliconed test tubes (100 × 15/16 mm.) and centrifuged at 1,000 *g* for 30 minutes. The supernatant wa. discarded, and the platelets were resuspended and washed once in 0·9% saline containing 1/10 by volume of triton 2% in 0·9% saline. The platelets were resuspended in 0·5 ml. 0·9% saline and quantitatively transferred to small flat metal cups (2·8 cm. diameter). The quantitative transfer was ascertained using 2 × 0·5 ml. saline for rinsing. The content (1·5 ml.) of the metal cups was air dried and then ready for the determination of the beta radiation. The weight of the dried content of a cup was 22 mg.

The thickness of radioactive substance is thus 3·6 mg./cm². The self-absorption in the sample can be neglected as this small thickness. Using this method, the activity was determined in the blood container immediately before the transfusion and in the recipients one and three hours after the transfusion was finished and subsequently daily or on every second day during the following four to eight days.

The cups were placed in an automatic sample changer and successively brought under a Geiger-Müller tube which was provided with a mica foil 1·4 mg./cm² thick. All the samples were automatically placed in the same position under the counter; the geometrical conditions were thus invariably identical. Four thousand pulses were counted and the corresponding time was automatically printed. The time could be read in hundredths of a minute. For the strongest samples a larger number of pulses was counted to determine the time with sufficient accuracy. The time was always five to 10 minutes. The error from reading the time is thus small, but the accuracy is influenced by the fact that the frequency of the current for the synchrotimer is constant only within 1%. Every sample was counted at least four times. Usually, counting was done at one-day intervals. In each counting, the calculated counts per minute were corrected for physical decay from the point in time at which platelet activity for a donor was first measured. Finally, the mean value of the corrected counts per minute was found.

Owing to the statistical fluctuation of the disintegrations the accuracy of 4,000 counts is about 1·5%. A mean value for several measurements was calculated, and the statistical error is therefore smaller than the figure given above. The resolution time of the Geiger-Müller counter is 100 μsec.; correction for losses due to the counter is thus less than 1%, even at the highest counting rates used in this investigation. On the whole, the accuracy of the activities observed was between 1 and 2%, which is sufficient for the present purpose.

The activity determined in the specimens was plotted on semilogarithmic paper against the time after the transfusion.

The platelet suspensions prepared were obviously not pure as there was slight contamination with red and white cells but it was generally found to be less than one red and one white cell per 1,000 platelets, and a control specimen containing five times as many red cells (but no platelets) had a negligible activity (one count/minute).

DETERMINATION OF PLATELET YIELD IN RECIPIENTS This figure is difficult to calculate accurately, but it was tried using the activity of the platelets in the bottle determined just before the transfusion, and the activity of the platelets in the first specimen drawn from the recipient one hour after the transfusion. (If the activity of the platelets from the patient was higher three hours after the transfusion, this activity was used.)

The platelet activity was determined in 20 ml. of 'plasma' produced from four test tubes, each containing 4·5 ml. whole blood (=A : 1 ml. EDTA 5% + 19 ml. blood) + 1 ml. EDTA and 3 ml. dextran. The haematocrit value was determined in A and corrected for added EDTA and trapped plasma (Mollison, 1956), and from this figure the plasma haematocrit value (H. pl.) was found. The exact volume of real plasma (x) in the 20 ml. was calculated using the following formula:—

$$x = \frac{\text{H.pl.} \times 100}{\text{H.pl.} + 89} \times \frac{20}{100}$$

From this figure and the determined platelet activity, the platelet activity per millilitre plasma was calculated for the donor (D) and the recipient (R). Then the total volume of plasma transfused (U), and the total plasma volume in the recipient (Y) were found, and the platelet yield was determined as $\frac{R \times Y \times 100}{D \times U}$. The total volume

of plasma (+ anticoagulant) transfused was calculated using the haematocrit value corrected for trapped plasma (Mollison, 1956) and using a total volume in the bottle of 500 ml. of blood plus the volume of anticoagulant added minus the volume taken for various tests. The total plasma volume in the recipient was found using the Wadsworth formula, which gives a total plasma volume = 43·1 × weight in kg. (Mollison, 1956). The plasma volume calculated was corrected for varying haematocrit in the recipients. Arbitrarily, a haematocrit level of 40% was used as the base line for the corrections. A difference in haematocrit of 1% was taken to be equal to 0·5 ml. plasma per kg. body weight using the diagram from Mollison (1956, p. 62).

The results of these calculations are shown in Table I.

RESULTS

Table I only includes 21 of the 24 experiments performed. Three of the recipients were excluded because the activity found was only minimal. The recipients excluded were (1) Case II B who received fresh ACD blood from an untreated glass bottle. This patient had been transfused on five previous occasions, and she had had three children and no abortions. (2) Case III B received 24-hour-old ACD blood from a Fenwal bag. The transfusion was stopped after 300 ml. because of a febrile reaction and chills. The patient had previously been transfused three times; she had had two normal pregnancies, (3) Case IV D received 15-hour-old ACD blood from a Fenwal bag. The patient had previously been transfused on three occasions, and she had never been pregnant.

As already mentioned, the results obtained with the 'pints' withdrawn from a single donor give a very good opportunity for comparing the recovery of transfused platelets in the recipients in relation to the various containers and anticoagulants used. The results obtained with the bottles of blood procured from the five different polycythaemic donors appear in Figs. 1 to 5 (Donor VI who only donated one 'pint' is excluded from these figures). No corrections have been made for varying plasma volumes in the recipients used and no corrections have been made for varying volumes of anticoagulant used in the different containers, all containing 500 ml. of donor blood. The results obtained in the various recipients all represent the platelet activity in equal volumes of plasma. No corrections for varying activity of the platelets from the donor have been made, but it appears from Table I that the platelet activity was

TABLE I

PLATELET YIELDS IN 21 EXPERIMENTS

				Bottle				*Platelet Antibodies*		*Recipient*				
Container	*Anticoagulant*	*Storage*	*Experiment No.*	*Haematocrit*	*Bottle Activity*	*Total Activity*	*Previous Transfusion Pregnancies*	*Complete*	*Incomplete*	*Haematocrit*	*Activity*	*Plasma Volume (ml.)*	*Total Activity*	*Recovery (%)*
Glass	ACD (Danish)	Fresh	I A			61,000	0/0	+	—				25,500	42
			III C	49	5,003	223,000	1/10	Not investigated		40	424	2,680	132,000	59
			V A	48	805	36,800	1/1	—	—	36	56	2,980	20,000	55
Glass (siliconed)	ACD (Danish)	Fresh	I B			51,000	0/0	—	—				37,800	74
			II D	47	680	30,400	1/3	—?	—	41	45	3,310	18,500	61
			IV A	53·5	2,974	127,000	1/1	Not investigated		35·5	176	2,280	47,900	38
			V C	42·5	836	39,800	0/3	+++	—	34	68	3,100	23,400	63
Plastic (Fenwal)	ACD, Fenwal	Fresh	I E			51,000	0/1	—	—				17,400	34
			II F	47	645	25,200	0/0	—	—	44	48	2,900	17,300	69
			III D	43	4,920	198,000	0/2	Not investigated		34	206	4,230	101,300	52
			IV C	49	3,060	118,800	3/0	Not investigated		40	235	2,240	65,500	56
			V B	48	1,113	44,800	0/4	—	—	36·5	57	2,540	17,400	39
Glass	EDTA, Fenwal	Fresh	I C			52,500	2/2	Not investigated					13,900	27
Glass (siliconed)			I D			45,500	0/9	—	—				16,500	36
Plastic (Fenwal)			I F			45,000	0/3	—	—				8,200	18
Glass	ACD (Danish)	24 hr.	VI	50	1,415	62,500	0/4	—	—	40	94	3,730	43,700	70
		15 hr.	IV B	49	2,617	116,000	1/13	Not investigated		40	124	2,570	39,600	34
		24 hr.	III A	53	4,828	203,000	2/6	Not investigated		43	161	3,370	63,200	31
		72 hr.	II A	55·5	688	28,700	0/1	—	—	39·5	14	2,310	3,950	14
Glass (siliconed)		72 hr.	II C	53·5	625	26,700	4/5	—	—	44	25	2,580	8,450	32
Plastic (Fenwal)	ACD, Fenwal	72 hr.	II E	45	770	30,500	0/3	—	—	27	10	3,500	3,950	13

I to VI are the sixth polycythaemic donors used. A to F are the different recipients who were transfused from the various donors.
Experiments using the six donations from donor I were all done with a slightly different technique working with the platelet activity per p latelet both in the bottles and in the recipients. The calculations used, therefore, differed from those in the rest of the experiments. The haema ocrit values are corrected for trapped plasma and added anticoagulant.

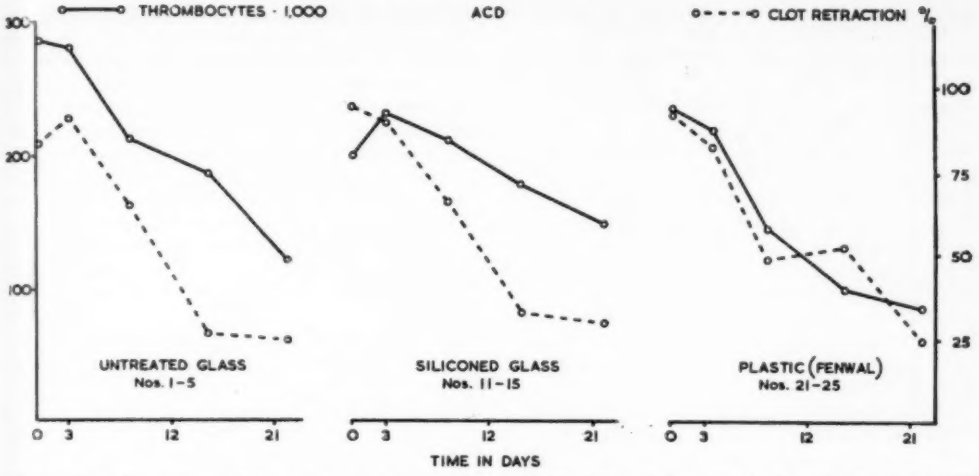

FIG. 1. *Thrombocyte survival in six recipients receiving 500 ml. blood from polycythaemic donor I treated with P^{32}. All experiments were performed with fresh blood using varying containers and anticoagulants.*

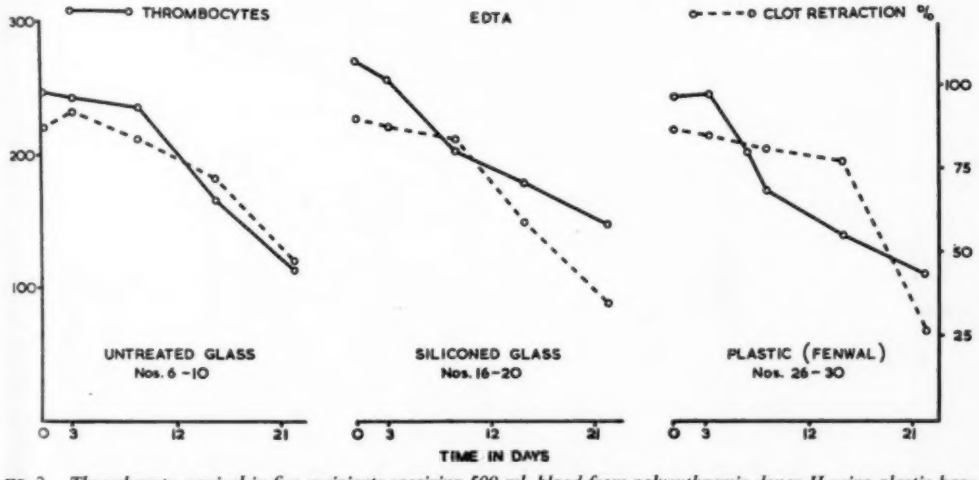

FIG. 2. *Thrombocyte survival in five recipients receiving 500 ml. blood from polycythaemic donor II using plastic bags, and untreated and siliconed glass.*

fairly constant during the experiments performed with blood donations from the respective donors, especially when it is taken into account that the volume of anticoagulant used is less in the Fenwal series than in the ACD (Danish) series. (The Fenwal ACD solution is 75 ml., the Fenwal EDTA solution 50 ml., and the Danish ACD solution used 150 ml.)

DISCUSSION AND CONCLUSION

The main conclusion which can be drawn from the experiments is that the container does not, to any appreciable degree, interfere with the recovery or the survival time of the thrombocytes if the blood is transfused during the first few days after the with-

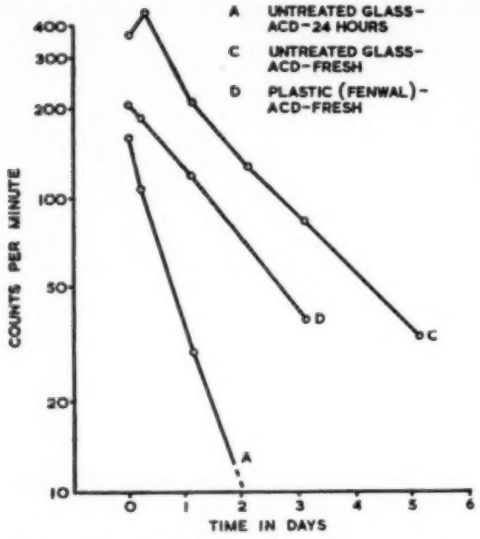

FIG. 3. *Thrombocyte survival in three recipients receiving 500 ml. blood from polycythaemic donor III using a plastic bag and untreated glass.*

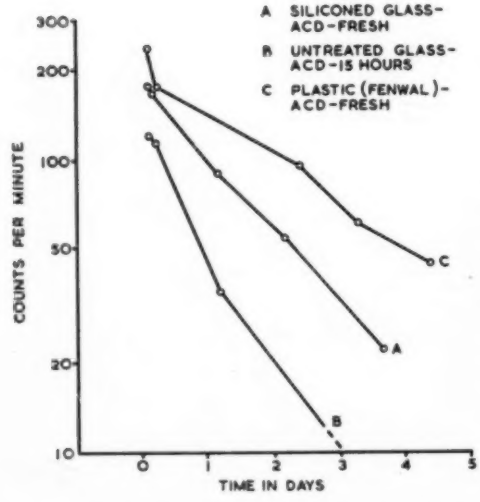

FIG. 4. *Thrombocyte survival in three recipients receiving 500 ml. blood from polycythaemic donor IV using a plastic bag and untreated and siliconed glass.*

drawal of the blood. Contrary to expectation neither siliconed glass nor plastic containers were necessary to secure a reasonable recovery of viable platelets.

In three experiments (Fig. 1, C, D, F) using EDTA (Fenwal formula) as anticoagulant we found that this anticoagulant was inferior to the ordinary ACD solutions. This finding was also contrary to our expectations as EDTA is to-day the anti-

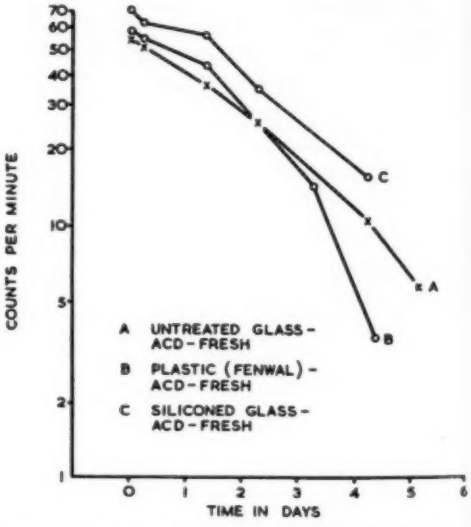

FIG. 5. *Thrombocyte survival in three recipients receiving 500 ml. blood from polycythaemic donor V using a plastic bag and untreated and siliconed glass.*

coagulant of choice in studies of platelets *in vitro*. It does not contain glucose, and it cannot be excluded that this may be of major importance. In all the EDTA experiments—and only in these experiments—did we find an initial 'dip' in the survival curves (Fig. 1, C, D, F), and this initial dip may be indicative of some damage to the platelets caused by the EDTA solution. EDTA is a very effective chelating agent, which may cause partly reversible damage to enzymes essential for the viability of platelets but this explanation is obviously only pure speculation. It should be mentioned that Adelson *et al.* (1957), who also determined the survival of platelets labelled *in vivo* with P^{32} using EDTA as anticoagulant, found an even more pronounced initial dip, but they made their first determination 30 minutes after the transfusion. In the present investigation the first determination was made one hour after the transfusion. The experiments with untreated and siliconed glass bottles and Fenval

bags using fresh ACD blood gave almost identical results. There is some variation in the platelet recovery in the various recipients (Table I), but the averages from the three groups of recipients are almost identical, namely 52% for untreated glass bottles, 59% for siliconed glass bottles, and 50% for Fenwal bags. In contrast to these relatively satisfactory results, the three EDTA experiments gave an average of only 27%. The number of experiments is limited, but nevertheless it seems justifiable to conclude that the Fenwal-EDTA solution used in the 'platelet pack' is in no way superior to the ordinary ACD-Fenwal bag or an ordinary ACD glass bottle.

It is generally claimed that blood should be fresh when given as a source of blood platelets, for example, to thrombocytopenic recipients. What is meant by 'fresh' is usually not exactly defined, which often results in a demand from the clinicians for delivery of the blood immediately after the bleeding. This has several practical drawbacks and causes the blood delivered to be less safe because of emergency handling (cross-match, serological tests, etc.). It is reasonable to believe that the platelet recovery will be maximal if the blood is given immediately after the bleeding, but the last five experiments in Table I show that the platelet recovery was quite satisfactory after a limited period of storage (average 45% after ≦ 24 hours of storage in untreated glass bottles), and even after storage for 72 hours some platelet activity could be found in the recipients. It is reasonable to believe that the use of silicone for longer periods of storage may be of importance, as it causes less activation of coagulation factors; a platelet recovery of 32% in experiment II C (Table I), in which blood was stored for 72 hours in a siliconed bottle, is in accordance with this suggestion.

The results of the recovery studies obtained with the various containers and anticoagulants after different periods of storage are summarized in Fig. 6.

As a consequence of the results obtained we now use blood which is less than 24 hours old as fresh blood, and in the absence of acute bleeding we use blood less than 72 hours old for thrombocytopenic recipients. Where a very high recovery of platelets is really essential, *e.g.*, for a bleeding thrombocytopenic patient we use, of course, fresh blood drawn immediately before the transfusion, as it must be assumed that even when the platelets are stored under optimal conditions at 4°C. their average survival time will decrease with the period of time during which they have been stored. For extracorporeal circulation we use blood drawn during the afternoon of the day before the operation, which with our very limited experience has been absolutely satisfactory.

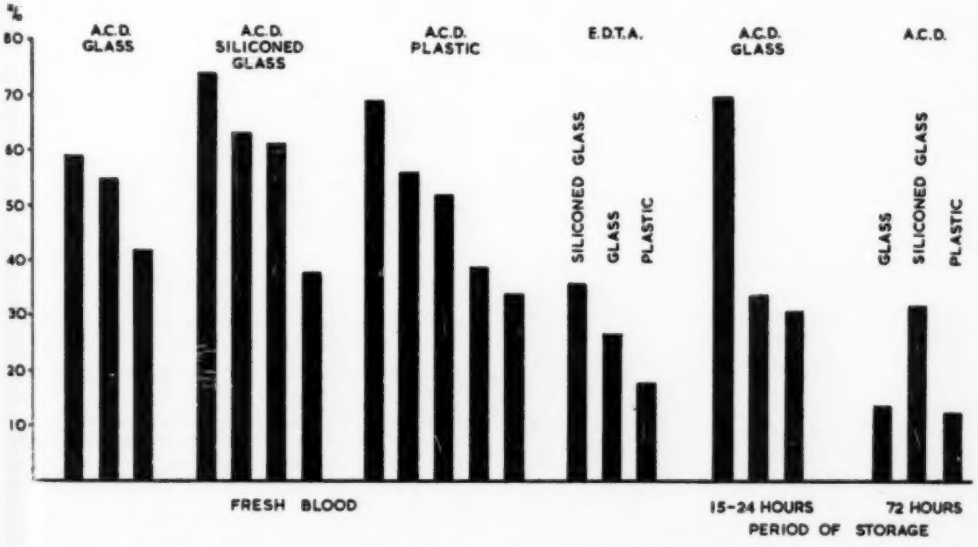

FIG. 6. *The various columns represent the percentages of the transfused platelets found in the recipients. The first four groups represent the results obtained using fresh blood. The last two groups represent the results obtained when the blood was transfused after varying periods of storage.*

It was not possible to find any explanation for the three failures in the experiments, but unfortunately no investigations were made for platelet antibodies in the three recipients. In experiment IIIB, the recipient suffered a febrile transfusion reaction. Neither erythrocyte nor complete leucocyte antibodies were found in her serum.

The results of the experiments reported are in disagreement with most previously performed work in this field, in which it is claimed that ordinary bank blood in untreated glass bottles is greatly inferior to that stored in plastic and siliconed equipment (Gardner, 1958; Stefanini and Dameshek, 1955; Tullis, 1953).

However, McIlvanie (1958) reported the results of an investigation in which he compared plastic bags, siliconed, and untreated glass bottles in transfusing fresh ACD blood to various patients and determined the platelet yield by counting the platelets. He found almost identical results for the various containers. The results he obtained with siliconed bottles were only slightly superior to those obtained with plastic bags and untreated glass bottles. The difference may not have been statistically significant, and thus McIlvanie's observations are in agreement with our results.

Most of the investigations in which superiority for plastic and siliconed equipment is claimed have been performed in America, where evacuated blood bottles are in common use. Such bottles cause more foaming during the bleeding, which, as pointed out by Stefanini and Dameshek (1955), may damage the platelets. However, this does not seem to be a plausible explanation for the poor results obtained with glass bottles, since even bleeding by gravity with automatic shaking causes some foaming.

Obviously, it cannot be excluded that the type of glass and silicone used may influence the results, but this problem was considered to be outside the scope of the present investigation.

It appears from Table I that some of the recipients had previously been transfused. Transfusions (and pregnancies) may cause iso-immunization against platelets, but it does not seem reasonable to assume that the transfusions given exerted any influence on the results obtained in a comparison of the various groups of containers.

In two of the recipients in whom the experiments were successful complete platelet 'agglutinins' were shown by Dausset's technique. Recipient IA had weak and recipient VC fairly strong agglutinins. These agglutinins were cold with an effect only at low temperature, and in spite of the presence of these agglutinins in the recipients normal platelet recovery and a normal platelet survival time were observed. These experiments are in agreement with two experiments performed by van Rood, Eernisse and van Leeuwen (1958, Figs. 1 and 3). These authors found a normal survival time in two patients with complete platelet agglutinins and no incomplete agglutinins. This is in harmony with the fairly frequent demonstration of complete, cold platelet agglutinins in normal subjects, and shows that the clinical importance of complete cold platelet agglutinins is still an unsolved problem.

A detailed discussion of the results obtained in relation to platelet life span will not be given, as this problem is outside the scope of the present investigation. It may, however, be stated that a $T\frac{1}{2}$ of two to three days was observed in most of the experiments in which fresh blood was used, while the use of stored blood resulted in some shortening (in most cases, $T\frac{1}{2}$ was about one day). The results obtained agree with those obtained by Adelson *et al.* (1957) who used a similar technique in two experiments. The limitations of the techniques in which isotope-labelled platelets are utilized will not be discussed, but reutilization and elution must obviously be taken into consideration. In using polycythaemic donors it must also be remembered that the life span of the platelets in these patients may either be decreased or increased (Alfos, Field, and Ledlie, 1959). However, these problems do not exert any influence on the results obtained in the present investigation or on the conclusion drawn in relation to the influence of the various containers on platelet recovery in blood transfusions.

Our thanks are due to Professor J. Bichel, chief of the Haematological Department, the Radium Centre for Jutland, and to Dr. K. Bjørn Jensen for their permission to use the polycythaemic patients in this investigation. The haematological work at the Radium Centre is supported by grants from the Anders Hasselbulch Anti-Leukaemia Foundation and Schepler and Wife's bequest, the Irma Foundation.

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Sodium, potassium, water, and haemoglobin in the packed red cells of severe thalassaemia

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SYNOPSIS Sodium, potassium, water, and the mean corpuscular haemoglobin concentration were determined in the packed erythrocytes of children with severe thalassaemia. The concentration of sodium in the packed red cells was higher than normal in a significant proportion of children with thalassaemia whereas potassium in the packed cells and sodium and potassium in the plasma were normal.

On average, the cell water content was a little higher and the mean corpuscular haemoglobin concentration a little lower than normal.

The cation concentrations in the packed cells of thalassaemia are compared with those in other anaemias. Similarities are pointed out between the sodium concentrations in the packed cells of thalassaemia and those from the foetus and children suffering from malnutrition.

Previous work has shown that the packed erythrocytes in many anaemias contained abnormal amounts of water and cations, chiefly potassium. The concentration of potassium in the packed cells was increased in microcytic anaemias, paralleling an increase in cell water (Maizels, 1936; McCance and Widdowson, 1956). In macrocytic anaemias the small increase in potassium reported by Maizels (1936) and Iyer (1958) was not confirmed by Bertcher and Meyer (1952) and was not always accompanied by a rise in the cell water.

The packed erythrocytes in spherocytic anaemias, congenital (Maizels, 1936; Selwyn and Dacie, 1954) or acquired (Selwyn and Dacie, 1954), have been shown to contain less potassium and less water than do normal cells.

In congenital, non-spherocytic haemolytic anaemia the concentration of potassium and the water content of the packed red cells were high in the cases with marked macrocytosis and reticulocytosis (type I) and normal in the cases with only occasional macrocytes and ovalocytes (type II) (Selwyn and Dacie, 1954). These deviations from normal in type II have been attributed to a high proportion of reticulocytes which are known to contain more potassium and more water than do mature erythrocytes (Granick, 1949; Keitel, Berman, Jones, and MacLachlan, 1955; Ponder, 1948).

In all these anaemias the concentration of sodium in the packed erythrocytes was normal.

The red cell potassium content was slightly low in sickle cell anaemia. During sickling the cells lost potassium and gained sodium without appreciable change in the total cation concentration or the water content (Tosteson, 1955; Tosteson, Carlsen, and Dunham, 1955; Tosteson, Shea, and Darling, 1952).

Experimental post-haemorrhagic anaemia in the dog and the rabbit resulted in a rise of the concentration of potassium and, sometimes, that of magnesium in the packed erythrocytes. A greater rise of these cations was observed in the haemolytic anaemia following the administration of phenylhydrazine and was thought to reflect the high proportion of reticulocytes in both these anaemias (Henriques and Ørskov, 1939).

No abnormality was found in the concentrations of sodium and potassium or the water content of the erythrocytes in the anaemia of myelosclerosis (Maizels, 1936).

In one patient with thalassaemia, Selwyn (1953) found that the loss of potassium from the red cells during incubation at 37°C. was greater than normal. Except for this information, however, nothing is

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known of the concentrations of sodium or potassium in the erythrocytes of thalassaemia. The present investigation records the results in thalassaemia.

MATERIAL AND METHODS

Venous blood was collected from children with severe thalassaemia and from normal children who served as controls. The group of controls consisted of children convalescing from diseases with no anaemia and no known effect on the concentrations of sodium or potassium in the plasma or the cells. Children taking drugs such as A.C.T.H. or cortisone were carefully excluded.

The concentration of sodium in the plasma and in the packed red cells was determined in 38 children with severe thalassaemia and 42 normal children. In 18 of the thalassaemic and 17 of the normal children plasma and cell potassium was also determined. Haematocrit readings were recorded and the haemoglobin concentration in whole blood determined in order to calculate the mean corpuscular haemoglobin concentration. The latter was also calculated in a further 18 anaemic and 19 normal children. Cell water was measured in 37 thalassaemic and 37 normal children. For greater accuracy anaemic and normal subjects were always investigated in pairs.

After a period of 10 to 12 hours during which no food was allowed, blood was drawn from the antecubital or jugular vein in the usual way and collected into centrifuge tubes containing lithium oxalate. (Lithium oxalate was preferred to other anticoagulants because almost all others contain sodium or potassium which could interfere with the estimation of these cations. On the other hand, it was thought unwise to use defibrinated blood because of the danger of causing haemolysis, especially in blood specimens from patients with thalassaemia, since erythrocytes are known to be mechanically fragile in this anaemia.) The amount of lithium used was not sufficient to give a reading on the flame photometer after dilution of the same order as that used for the blood samples. The plasma was separated as quickly as possible in order to prevent exchange of sodium and potassium between the plasma and the cells.

Sodium and potassium in the plasma and the cells were estimated according to the methods described by Selwyn and Dacie (1954) except that suitable adjustments were made for direct analysis on an E.E.L. flame photometer, which was calibrated with standard dilutions containing 0·3, 0·5, 0·7, and 1·0 mg. % of sodium or potassium and the samples made up to a suitable volume to give readings very near those of one of the above standard solutions.

At the beginning of the present study it had been decided to correct the concentrations of cell sodium and potassium for trapped intercellular plasma according to the results of Chaplin and Mollison (1952). Since, however, these authors constructed their calibration curves from normal blood specimens and, moreover, found that plasma trapping was slightly different in the presence of abnormal erythrocytes, it was later thought wiser to construct our own calibration curves for both normal and thalassaemic children. Trapped plasma was, therefore, measured according to the method of Chaplin and Mollison in 23 children with severe thalassaemia and 33 normal children, and calibration curves were constructed from 72 measurements of thalassaemic and 92 measurements of normal blood. These results are published in detail elsewhere.

In 15 of the anaemic and 18 of the normal children investigated the trapped plasma was estimated at the same time as the cell sodium. In these cases the samples of packed erythrocytes to be used for cell sodium estimations were obtained after centrifuging whole blood at 1,500 *g* (3,000 r.p.m. for 30 min., radius 15 cm.) in Wintrobe haematocrit tubes. This was done in order to produce exactly the same conditions for both cell sodium and trapped plasma estimations. The results obtained by this method for cell sodium were almost identical with those obtained by the method of Selwyn and Dacie (1954). In these cases cell sodium was corrected using each child's own value for trapped plasma. In the remaining patients of the present study, in whom the amount of trapped plasma had not been measured, corrections were made using our calibration curves.

The accuracy of the estimations of sodium and potassium was tested by treating standard solutions of these cations at the same time and in exactly the same way as the samples of plasma, whole blood, or packed red cells. Recoveries were 99·8 to 106 % (mean 102·7 %) for sodium and 96·8 to 106% (mean 102·6 %) for potassium. When standard solutions were added to samples of plasma, whole blood, or packed cells, recoveries were 96 to 109 % (mean 102 %) for sodium and 98 to 104 % (mean 101%) for potassium.

Cell water was measured by drying to constant weight at 100° to 105°C. weighed samples of packed cells prepared as for cell sodium determinations.

Haemoglobin in whole blood was estimated according to the Sanford, Sheard, and Osterberg oxyhaemoglobin method (Kolmer, Spaulding, and Robinson, 1951) and the proportion of alkali-resistant haemoglobin according to the method of Singer, Chernoff, and Singer (1951). Haematocrit readings were recorded after centrifuging at 3,000 r.p.m. for 30 min. (radius 15 cm.) in Wintrobe tubes.

Whenever possible estimations were made in duplicate.

RESULTS

The normal concentrations of sodium and potassium in the packed erythrocytes were, on the average, close to those found by Maizels (1936), McCance and Widdowson (1956) and Selwyn and Dacie (1954) in normal adults (Table I). Erythrocytes in infants up to 2 years of age have been shown to contain more sodium but no more potassium than that accepted as normal for adults (Overman, Etteldorf, Bass, and Horn, 1951), although they were of adult composition during the first few hours after birth (McCance and Widdowson, 1956; Overman *et al.*, 1951). In the present series of normal children the concentration of sodium in the packed erythrocytes was about

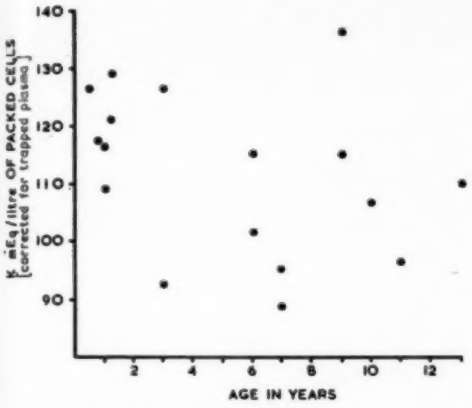

FIG. 1. *Relationship between potassium in the packed erythrocytes and age in normal children.*

the same in both the babies aged 2 to 24 months and the older children but that of potassium tended to be a little higher in the infant age group (Fig. 1).

Table I shows that the concentration of sodium in the packed red cells was, on the average, higher in the children with thalassaemia. In spite of some degree of overlapping, the mean concentration of sodium was already appreciably higher before the obtained values were corrected for trapped intercellular plasma, but the difference between the anaemic and the normal subjects, expressed as a percentage, was even greater after correction. The difference between the means of the two groups was found to be statistically significant both before and after correction (see Appendix).

As demonstrated in Table II, in 13 out of 38 children with severe thalassaemia (Cases 2, 3, 5, 6, 10, 13, 24, 27, 28, 29, 30, 31, and 32) the sodium concentration in the packed red cells exceeded the highest concentration observed in the normal children. In eight of these anaemic children (Cases 3, 5, 24, 28, 29, 30, 31, and 32) the excess ranged from 25 to 100%. It should be pointed out, however, that when each anaemic child was compared with its own control, there were another seven thalassaemic children in whom the concentration of sodium in the packed cells was higher than normal by 30 to 90% (Cases 9, 14, 16, 17, 23, 34, and 35). This brings the total number of thalassaemic children with high sodium concentrations to 20 (52%). Of the remaining 18 cases, concentrations were about the same as those of their controls in 17. There was one child (Case 20) in whom the concentration of sodium was considerably less than that of its control.

In nine cases of thalassaemia the cell sodium content was determined twice over a period of a few days to 12 months (Table II). In three of these children (Cases 1, 7, and 14) the concentration was pretty constant. In Case 14 the second estimation was performed a few days after splenectomy but the concentration of sodium remained unchanged in spite of an approximately twofold increase in whole blood haemoglobin and the haematocrit reading. In the remaining children with thalassaemia who were examined twice, the cell sodium varied but it should be pointed out that re-examination generally

TABLE I

CONCENTRATIONS OF SODIUM AND POTASSIUM AND WATER CONTENTS OF PLASMA AND PACKED ERYTHROCYTES AND AMOUNT OF PLASMA TRAPPED WITHIN THE RED CELL COLUMN IN NORMAL AND THALASSAEMIC CHILDREN

	Plasma				*Packed Erythrocytes*						
	Na (mEq./l.)	*K (mEq./l.)*	*Na+K (mEq./l.)*	*Water (g./100 g.)*	*Na (mEq./l. Uncorrected for Trapped Plasma)*	*K (mEq./l. Uncorrected for Trapped Plasma)*	*Trapped Plasma (% of Red Cell Column)*	*Na (mEq./l. Corrected for Trapped Plasma)*	*K (mEq./l. Corrected for Trapped Plasma)*	*Na+K (mEq./l. Corrected for Trapped Plasma)*	*Water (g./100 g.)*
Normal children (6 mth. to 13 yr.)											
Maximum	156·4	5·82	161·2	92·5	19·18	132·5	3·55	15·95	136·8	147·9	66·9
Minimum	121·2	4·12	138·4	90·3	11·40	86·4	2·55	6·90	89·1	96·8	55·3
Mean	135·6	5·03	146·7	91·3	15·17	108·6	3·10	11·30	112·3	123·0	62·7
S.D.	±8·9	±1·45	±6·9	±0·5	±2·17	±13·6	±0·27	±2·35	±13·7	±14·8	±2·3
No. of observations	42	17	17	28	42	17	42	42	17	17	37
Children with severe thalassaemia (2 mth. to 13 yr.)											
Maximum	144·6	5·87	148·1	92·6	34·97	130·8	4·95	32·13	135·9	148·9	69·6
Minimum	124·8	4·12	135·4	90·9	13·08	102·4	2·67	8·43	103·7	120·7	58·3
Mean	134·0	4·75	141·3	91·5	19·86	115·0	3·82	15·81	118·3	133·1	64·9
S.D.	±5·0	±1·73	±3·6	±0·5	±5·1	±8·3	±0·45	±5·4	±9·1	±8·4	±2·1
No. of observations	38	18	18	24	38	18	38	38	18	18	37

TABLE II

CONCENTRATION OF SODIUM IN PACKED ERYTHROCYTES OF NORMAL AND THALASSAEMIC CHILDREN[1]

Normal			*Thalassaemia*			
Series No.	*Age*	*Sodium*	*Series No.*	*Age*	*Sodium*	
1	10 yr.	13·34	1a	9 yr.	10·07	10·17
2	9 yr.	7·90	1b	10 yr.		9·98
3	7 yr.	7·75	2	4 yr.	17·52	
4	3 yr.	6·90	3[2]	12 mth.	19·86	
5	12 mth.	11·86	4[2]	3 mth.	11·52	
6	6 yr.	13·92	5a[2]	6 mth.	22·49	25·14
7	12 yr.	12·67	5b	11 mth.		19·85
8	14 mth.	11·49	6	12 yr.	17·23	
9	11 yr.	11·19	7a	11 mth.	12·55	12·49
10	2 yr.	9·70	7b	14 mth.		12.60
11	7 yr.	12·43	8[2]	10 mth.	12·12	
12	12 mth.	8·24	9	6 mth.	12·25	
13	13 yr.	8·91	10	3 yr.	17·04	
14	15 mth.	12·57	11	2 yr.	14·48	
15	6 mth.	10·85	12	3 yr.	12·61	
16	9 yr.	11·12	13	10 mth.	17·13	
17	9 yr.	9·22	14a	2 yr.	13·97	13·94
			14b[2]	2 yr.		14·02
18	9 mth.	13·07	15	2 yr.	13·26	
19	6 yr.	7·85	16	2 yr.	15·11	
			17	12 mth.	13·55	
20	3 yr.	12·77	18	3 yr.	11·56	
21	10 yr.	9·96	19	10 yr.	9·38	
22	2 yr.	12·71	20	2 yr.	8·43	
23	2 yr.	7·66	21a	14 mth.	12·55	9·20
24	3 yr.	13·79	21b	2 yr.		15·90
25	2 yr.	10·06	22	2 yr.	9·14	
26	10 yr.	10·40	23	4 yr.	13·49	
27	17 mth.	9·14	24a	2 yr.	20·78	25·23
28	4 yr.	10·76	24b	3 yr.		16·34
29	3 yr.	13·42	25	3 yr.	14·38	
30	12 mth.	15·95	26	14 mth.	14·88	
31	12 mth.	10·55	27[2]	11 yr.	17·97	
32	12 yr.	15·23	28a	13 yr.	22·91	26·23
33	12 yr.	13·92	28b	13 yr.		19·59
			29a	19 mth.		26·98
34	7 yr.	9·70	29b	19 mth.	32·13	42·32
35	9 mth.	13·06	29c	19 mth.		27·10
			30	5 yr.	28·24	
36	8 mth.	14·85	31	14 mth.	29·87	
			32a	7 mth.	21·05	23·38
37	7 mth.	9·90	32b	7 mth.		18·72
38	4 mth.	15·23	33	2 mth.	14·04	
39	5 yr.	8·54	34	6 yr.	14·68	
40	4 yr.	10·82	35	4 yr.	14·08	
28	4 yr.	10·99	36	3 yr.	12·88	
41	4 yr.	11·70	37	6 yr.	13·23	
42	13 mth.	13·63	38	11 mth.	14·84	

[1]Results are expressed in mEq./l. corrected for trapped plasma.
[2]Recent blood transfusions
[3]After splenectomy

confirmed the high or normal values which had been obtained initially.

The present series of severe thalassaemia included two pairs of siblings (Cases 16 and 17 and Cases 25 and 26, Table II). The concentrations of cell sodium were almost identical and their haematological data showed little if any variance.

The mean concentration of potassium in the packed erythrocytes of the children with severe thalassaemia was about the same as that of the normal children and so were the concentrations of sodium and potassium in the plasma (Table I see Appendix). In some of the normal and some of the anaemic subjects the concentration of potassium in the packed erythrocytes was high.

The mean corpuscular concentration of haemoglobin in the normal children (Table III) was, on the average, very close to that reported by other investigators (Wintrobe, 1956) but nine children had values of 38% or over. This finding, together with the high concentration of cell total base observed in some of the cases, will be discussed later.

TABLE III

HAEMATOLOGICAL DATA FROM NORMAL CHILDREN AND CHILDREN WITH SEVERE THALASSAEMIA

	Haemoglobin (g./100 ml.)	*Alkali-resistant Haemoglobin (%)*	*Volume Packed R.B.C.s (ml./100 ml.)*	*M.C.H.C. (%)*
Normal children 6 mth. to 13 yr.				
Maximum	15·56	—	43·5	41·5
Minimum	9·43	—	30·5	28·5
Mean	12·73	—	35·9	35·4
S.D.	±1·2	—	±3·8	±2·8
No. of observations	55	—	61	55
Children with severe thalassaemia 2 mth. to 13 yr.				
Maximum	13·14[1]	90	33·5[2]	46·9[1]
Minimum	2·10	24	9·0	16·5
Mean	6·12	55	18·1	33·8
S.D.	±2·07	—	±4·9	±4·6
No. of observations	56	56	56	56

[1]Recent blood transfusions
[2]After splenectomy

In the children with severe thalassaemia the mean corpuscular haemoglobin concentration was, on the average, only very slightly lower than normal (Table III). In most of them it was normal or slightly reduced, the reduction being considerable in only five cases. On the contrary, anaemic red cells sometimes contained a concentration of haemoglobin which exceeded the highest value obtained from the normal children.

That the mean corpuscular haemoglobin concentration was, on the average, only slightly reduced in the children with thalassaemia is confirmed by the fact that there was an equally slight increase in the mean cell water of these children. This was 62·7% w/w in the normal children and 64·9% w/w in thalassaemia. If the specific gravity of the solids is 1·3 (a likely figure), their volume must have been 28·7 ml. and 27·0 ml. and cell water $62{\cdot}7 \times \frac{100}{62{\cdot}7+28{\cdot}7} =$ 68·6% w/v and $64{\cdot}9 \times \frac{100}{64{\cdot}9+27{\cdot}0} = 70{\cdot}6\%$ w/v respectively. The figures for both the normal and the

anaemic children are probably a little low (Maizels, 1936; McCance and Widdowson, 1956; Selwyn and Dacie, 1954) but it is obvious that the difference between the two groups is small.

Table IV gives the concentrations of sodium, potassium, and total base per litre of cell water. It is clear that correction for cell water did not alter our findings about the cation concentrations of the packed erythrocytes in severe thalassaemia.

TABLE IV

CONCENTRATIONS OF SODIUM AND POTASSIUM IN PACKED ERYTHROCYTES OF NORMAL AND THALASSAEMIC CHILDREN

		Na	*K*	*Na + K*
		(mEq./l. of cell water corrected for trapped plasma)[1]		
Normal	Maximum	23·2	199·0	215·0
	Minimum	10·0	129·0	141·0
	Mean	16·4	163·0	180·0
Thalassaemia	Maximum	46·92	189·5	210·0
	Minimum	11·94	150·5	172·9
	Mean	22·39	167·5	189·9

[1]Corrections for cell water were made using the calculated values of 66·6% w/v for normal and 70·6% w/v for children with thalassaemia

DISCUSSION

CONCENTRATIONS OF SODIUM AND POTASSIUM IN THE RED CELLS The results obtained from 38 children with severe thalassaemia show that the concentration of sodium in the packed erythrocytes was, on the average, higher than normal and it is of special interest that similar studies of the erythrocytes in other anaemias have yielded normal sodium concentrations (Bertcher and Meyer, 1952; Henriques and Ørskov, 1939; Maizels, 1936; McCance and Widdowson, 1956; Selwyn and Dacie, 1954, Tosteson *et al.*, 1952).

The erythrocytes analyzed consisted of a mixture of packed cells and trapped intercellular plasma. Since so much more sodium is contained in the plasma than in the packed cells, it was necessary to correct the sodium values obtained for trapped plasma, for it could be suggested that the higher concentration of sodium in thalassaemia had resulted from a greater amount of plasma trapped between the centrifuged red cells of this anaemia. The investigation of 23 of the children with severe thalassaemia in the present series revealed a somewhat greater amount of plasma remaining with the erythrocytes after centrifugation (Table I), but this was not sufficient to account for the increased concentration of sodium which was found in their packed cells. The sodium concentrations, therefore, remained higher than normal even after correction for trapped plasma.

Because of the wide spread in the concentrations of sodium in the packed cells of the children with thalassaemia, the question was raised whether cell sodium was related to the severity of the anaemia. Although higher concentrations tended to be found in patients who appeared to be severely ill, there was no correlation between the sodium concentration and the degree of the anaemia (Figs. 2 and 3).

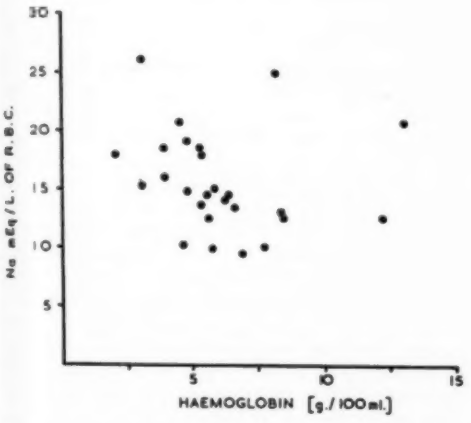

FIG. 2. *Relationship between sodium in the packed erythrocytes and haemoglobin in the whole blood in children with severe thalassaemia. (The figures for sodium have been corrected for trapped plasma.)*

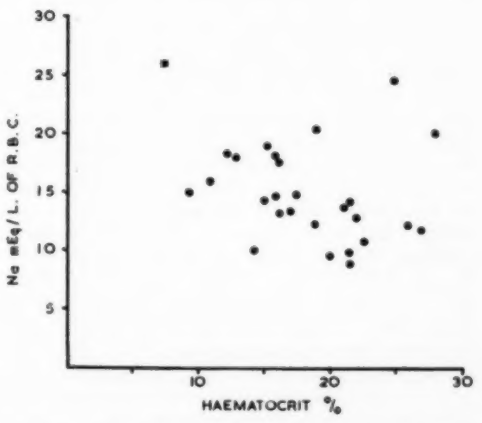

FIG. 3. *Relationship between sodium in the packed erythrocytes and the packed cell volume in children with severe thalassaemia. (The figures for sodium have been corrected for trapped plasma.)*

Cases 3, 4, 5a, 8, and 27 (Table II) had a relatively high haematocrit reading and haemoglobin concentration, the result of recent blood transfusions. These children were severely anaemic and had, therefore, been transfused with large quantities of blood before they were examined. The presence of normal erythrocytes would have been expected to lower the sodium concentrations in the packed cells. Yet, the latter were appreciably high in Cases 3, 5a, and 27. This observation, together with the fact that only very few cases have been examined under such conditions, makes the effect of blood transfusions on the concentration of sodium in the packed red cells very difficult to interpret.

An important question was whether the high sodium concentrations in the packed cells of many children with severe thalassaemia reflected an increase in the sodium content of the individual erythrocyte. Conclusions about the contents of individual erythrocytes can only be reached if their mean volume is known. Thus, with small erythrocytes each unit volume of packed cells includes a large number of cells; hence, if the sodium content of a small cell be the same as that of a normal cell, then a unit volume of small packed cells will be found to contain more sodium than the same unit of normal packed cells and the opposite is true of large cells. The mean cell volume was not calculated in the present study because of the large error involved in the enumeration of erythrocytes, but thalassaemia is known to belong to the microcytic anaemias. Nevertheless, it is very unlikely that the high concentration of sodium in the packed cells of many children with thalassaemia had resulted from a small mean cell volume, since the packed cells of microcytic anaemias other than thalassaemia have been shown to contain normal concentrations of sodium (Maizels, 1936; McCance and Widdowson, 1956). Furthermore, if microcytosis were responsible for the high concentrations of sodium, it would be very difficult to explain why it did not cause a proportional rise in the concentration of potassium in the packed cells of thalassaemia. It therefore appears that the average red cell in the thalassaemic children with high sodium concentrations in the packed cells did contain more sodium than normal. Unfortunately, information about the intracellular composition of the individual erythrocyte may be provided from the calculation of the mean cell volume only when all the cells are of similar size. In view of the marked variation in the size and shape of the erythrocytes in thalassaemia, one should be able to sort them into their various sizes and examine each size separately in order to estimate its intracellular composition. When this can be done, it may be possible to determine whether it is all the red cells in this anaemia or only a certain cell population which contains an increased amount of sodium. Until then, one must perforce express results and compare them with those obtained from other erythrocytes in terms of concentration per unit volume of packed cells.

In these terms the mean cell sodium in thalassaemia was higher than that of normal children, higher than that of other anaemias, but comparable with that of the foetus and the severely malnourished pig (McCance and Widdowson, 1956). This similarity between thalassaemic red cells on the one hand and those from the foetus and malnourished pigs on the other is interesting, because both thalassaemic and foetal erythrocytes contain a large proportion of alkali-resistant (foetal ?) haemoglobin and malnutrition is known to be a common symptom of severe thalassaemia.

The potassium concentration in the packed erythrocytes was approximately the same in both the normal and the thalassaemic children. This again differentiates thalassaemia from other types of anaemia in which it was the potassium not the sodium concentration which was abnormal (Henriques and Ørskov, 1939; Maizels, 1936; McCance and Widdowson, 1956; Selwyn and Dacie, 1954; Tosteson *et al.*, 1952).

As already mentioned, in some of the normal and some of the anaemic children of the present study the concentration of total base in the packed erthrocytes was high. Several figures for the mean corpuscular haemoglobin concentration were also high, whereas the average cell water in normal children was a little lower than that reported by other workers (Maizels, 1936; McCance and Widdowson, 1956; Selwyn and Dacie, 1954). These findings may all be tied up with the use of lithium oxalate as an anticoagulant which must have caused cells to shrink by raising the osmotic equivalence of the plasma. The degree of cell dehydration would vary if the lithium oxalate did not always dissolve completely and this would explain why some but not all of the figures for total base or the mean corpuscular haemoglobin concentration were high. Nevertheless, it is very unlikely that lithium oxalate caused a greater dehydration in thalassaemic red cells than in normal cells. A greater cell shrinkage in thalassaemia would certainly explain the higher concentration of sodium found in the packed cells, but, were the increase in sodium the result of cell dehydration, the latter should have caused a proportional rise in the concentration of potassium.

MEAN CORPUSCULAR CONCENTRATION OF HAEMOGLOBIN The normal or only slightly reduced mean corpuscular concentrations of haemoglobin found

in the present group of thalassaemia are not in general agreement with those previously observed by other investigators. In thalassaemia the mean corpuscular haemoglobin concentration is usually low (Lie-Injo Luan Eng, Kho Lien Keng, Liem Djwan Lioe, and Oei Oen Bie, 1959; Marmont and Bianchi, 1947; Minnich, Supa Na-Nakorn, Soodsarkon Chongchareonsuk, and Su-Ed Kochaseni, 1954; Sturgeon and Finch, 1957; Wintrobe, 1956) but it has already been demonstrated that it may be relatively normal (Lehmann, 1959; Lie-Injo Luan Eng *et al.*, 1959; Mathoth, Shamir, and Freundlich, 1955). This controversy may be due to differences in the morphology of the erythrocytes in the subjects studied by each group of investigators and similar differences may partly explain the wide spread in the values obtained from the present series.

More puzzling is the fact that the mean corpuscular haemoglobin concentration was higher than normal in a number of our cases. With the exception of spherocytes and ovalocytes, which have occasionally been shown to contain concentrations of haemoglobin which were slightly raised (Maizels, 1936; Selwyn and Dacie, 1954; Wintrobe, 1956), red cells with abnormal concentrations of haemoglobin are 'hypochromic' not 'hyperchromic'. The reason for this occasional hyperchromia of thalassaemic erthrocytes is obscure. In spherocytes the higher haemoglobin concentration was accompanied by a lower cell water content and it is conceivable that this may be true of some cases with severe thalassaemia.

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APPENDIX

Statistical treatment of the results of the present investigation (C. Callerghis, statistician, Oxford University) yielded the following results:

CONCENTRATION OF SODIUM IN PACKED RED CELLS The difference between the means of the thalassaemic and the normal children was highly significant. For values uncorrected for trapped intercellular plasma the Behrens test[1] gave $d = 5{\cdot}25$, $\theta = 32°$, with $n_1 - 1 = 37$ and $n_2 - 1 = 41$ degrees of freedom. The corresponding Sukhatmé table value is not greater than 3·0 at the 0·01 level of significance. For values corrected for trapped plasma $d = 4{\cdot}72$, $\theta = 32°$ with $n_1 - 1 = 37$, $n_2 - 1 = 41$ degrees of freedom. The Sukhatmé table value is about 3·0 at the 0·01 level of significance.

CONCENTRATION OF POTASSIUM IN PACKED RED CELLS The difference between the means of the two groups examined was not significant. Before correction for trapped plasma $t = 1{\cdot}68$, after correction $t = 1{\cdot}53 < 2{\cdot}030$ ($P = 0{\cdot}05$).

CONCENTRATION OF SODIUM AND POTASSIUM IN PLASMA For sodium $t = 0{\cdot}79$ and for potassium $t = 2{\cdot}109 > 2{\cdot}04$ at the 0·05 level of significance.

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION The small difference between the means of the two groups examined was statistically significant. The Behrens test gave $d = 2{\cdot}23$, $\theta = 31°$ with $n_1 - 1 = 55$, $n_2 - 1 = 54$. The corresponding Sukhatmé table value is not greater than 1·96 at the 0·05 level of significance.

CELL WATER The difference between the means of the two groups examined although small was highly significant for $t = 4{\cdot}294 > 2{\cdot}648$ at the 0·01 level of significance.

RELATIONSHIP BETWEEN MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION AND CELL WATER An inverse correlation was found between these two variables. In the group of thalassaemic children $r = -0{\cdot}325$ the Fisher and Yates (1938) table value for $n - 2 = 35$ degrees of freedom at the 0·05 level of significance.

In the group of normal children $r = -0{\cdot}603 > -0{\cdot}325$, the Fisher and Yates table value for $n - 2 = 35$ degrees of freedom at the 0·05 level of significance.

[1]The Behrens test has been applied throughout the present study whenever the variances of the concerned groups were shown to differ significantly (F test).

J. clin. Path. (1961), **14**, 644

The development of the serum protein pattern in Africans

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SYNOPSIS The serum protein of African and European mothers, African and European umbilical cord blood, and African children up to 6½ years of age have been investigated. African umbilical cord blood contains significantly higher concentrations of γ globulin than European umbilical cord blood. Significant differences were not found in the other protein fractions. The level of γ globulin in the serum of African mothers is significantly higher than the corresponding level in European mothers. Significant differences were not observed in the other protein fractions. The changes in serum proteins in pregnancy in African women follow the pattern described in European women. Close correlation was demonstrated between the level of maternal and cord blood γ globulin. The significance of this finding has been discussed.

It is concluded that hereditary factors cannot be excluded until further information on the level of γ globulin of Europeans and other races subjected to the conditions of life of the average African is available.

Although it is generally accepted that repeated contact with the malaria parasite and possibly other pathogenic organisms is the immediate stimulus for healthy Africans to maintain a higher serum gamma globulin concentration than Europeans (Holmes, Stanier, and Thompson, 1955; McGregor, Gilles, Walters, Davies, and Pearson, 1956; Edozien, Boyo, and Morley, 1960), recent comparative studies of the serum proteins in different population groups have also suggested that the difference in serum γ globulin concentration between ethnic groups may have a genetic basis (Rawnsley, Yonan, and Reinhold, 1956; Vera and Roche, 1956; Edozien, 1957, 1958; Bakker, Bliek, and Luyken, 1957; Comens, 1957; Curnow, 1957; Keltz and Comstock, 1959). The immunological and hereditary factors are not mutually exclusive, because it is possible that in certain environments, the process of natural selection may operate in favour of individuals who are able to provide high levels of circulating antibody and thus possess a high natural resistance to certain endemic diseases.

In order to further our understanding of the causes of difference between the mean γ globulin levels of ethnic groups it is important to ascertain whether this difference is present at birth, and if not, to determine the age at which it first becomes manifest.

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Symul (1950), using a chemical method of analysis, found all fractions in African cord blood close to the values obtained in European cord blood. Van Oye and Charles (1951), also using a chemical method, found all fractions, but especially the β and γ globulin, lower in the cord blood of African babies as compared with that of African adults. Bersohn, Wayburne, Hirsch, and Sussman (1954), again employing a chemical method, found no significant differences between the albumin and total globulin levels of European and African infants at birth, but the γ globulin level of the African was significantly higher than that of the Europeans. Stanier and Thompson (1954) measured the serum proteins of African umbilical cord blood, maternal blood, and the blood of young African infants in Uganda by microelectrophoresis (Antweiler) and reported that the main differences as compared with corresponding data on white persons were significantly lower mean levels of albumin and β globulin: the mean α and γ globulin levels at birth were found to be the same as for white persons. Their results for the African infants were compared with values obtained by Longsworth, Curtis, and Pembroke (1945) for American babies. Electrophoretic data from different laboratories are unfortunately notorious for their wide variation, especially if different analytical techniques are employed, and the conclusions of Stanier and Thompson (1954) must be viewed

against this background. Close (1955) found that the γ globulin concentration was lower in the newborn African child than in the European child and related this to poor nutrition. Thompson (1956) investigated the serum proteins in Uganda Africans under 18 months of age and concluded that the serum protein pattern in the first month of life is similar to that found in Europe and America and that the characteristic adult pattern becomes evident towards the end of the first year.

The reports available at present about the serum proteins of newborn Africans as compared with newborn Europeans thus provide very contradictory information and there is therefore need for further study, in particular for study of the newborn of both races by the same observers using identical methods. This has been done in the present investigation, the results of which are presented here.

MATERIALS AND METHODS

The human subjects studied are: (1) Seventy-five healthy adult women in Ibadan, being part of the group of 350 adult Nigerians previously reported; (2) 68 Nigerian women in the second and third trimesters of normal pregnancy; (3) 71 umbilical cord bloods of Nigerian babies and the bloods of 67 of their mothers collected within 12 hours of delivery (some of the children were followed at monthly intervals up till 6 months); (4) 20 umbilical cord bloods of European babies and the bloods of 12 of their mothers also collected within 12 hours of delivery; (5) 113 children aged $4\frac{1}{2}$ to 26 months at Imesi, a village approximately 100 miles north-east of Ibadan. These were part of the group of 211 children previously reported (Edozien *et al.*, 1960) but as 98 of these children were protected from malaria by monthly doses of pyrimethamine (Daraprim) they have been excluded from the present investigation. (6) One hundred and fifty-six children aged $2\frac{1}{2}$ to $6\frac{1}{2}$ years at Ilesha, a town very close to Imesi.

Blood was collected by femoral vein puncture from the children and from an antecubital vein in the adults.

The handling of the specimens and the method for estimating total proteins and the protein fractions were those described previously (Edozien *et al.*, 1960). Immuno-electrophoresis was performed according to the procedure of Grabar (1959). Difco bactoagar was used without further purification. The unstained slides were photographed immersed in tap water: they were then stained with 1% bromophenol blue for two minutes and washed for 10 minutes in 10% acetic acid followed by tap water for 18 hours.

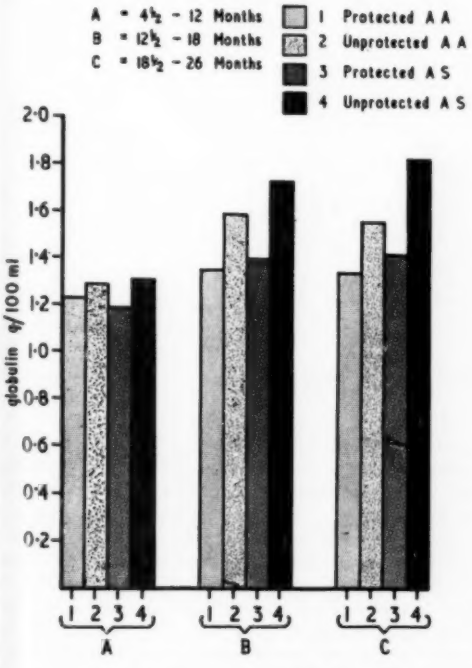


FIG. 1. *The effect of malaria prophylaxis on the serum γ globulin concentration in children (drawn from the figures in Edozien* et al.).

RESULTS

The mean values, together with standard deviations for the total serum proteins and the protein fractions in healthy adult women in Ibadan, and in normal pregnancy are given in Table I. Compared with adult Europeans, the non-pregnant Nigerian women have lower albumin and higher γ globulin concentrations but this is the usual pattern in symptom-free Nigerians. In pregnancy there is a progressive fall in the concentration of total protein and of albumin and a rise in the concentration of α_1, α_2, and β globulins. These changes are most marked at term. There is also a drop in the γ globulin concentration towards the end of pregnancy. These changes are similar to those which take place in pregnant European and American women (Poidevin, 1954; Mack, 1955).

Table II shows the mean values for the total proteins and the protein fractions in the serum of umbilical cord blood of African and European babies and in the blood of their mothers taken on the day of delivery. The individual values are represented graphically in Figs. 2 to 7. The statistical significance of the results is recorded in Table III. The results for European mothers and for the cord blood of European babies agree very closely with the results of others (Longsworth *et al.*, 1945; Beach, Coryell, Moyer, Robinson, Schoeb, Wiseman, Macy, and Mack, 1952; Moore, Du Pan, and

TABLE I

SERUM PROTEINS IN NON-PREGNANT WOMEN AND IN NORMAL PREGNANCY

	Non-pregnant Women (75)		*Second Trimester (24)*				*Third Trimester (39)*				*Term[2] (67)*			
	Mean	*S.D.*	*Mean*	*S.D.*	*t*[1]	*p*[1]	*Mean*	*S.D.*	*t*[1]	*p*[1]	*Mean*	*S.D.*	*t*[1]	*p*[1]
Total proteins	7·02	0·41	6·75	0·59	2·5	0·01<p<0·02	6·80	0·73	2·3	0·01<p<0·02	6·35	0·57	7·5	p<0·01
Albumin	3·50	0·31	2·66	0·46	10·1	p<0·01	2·61	0·35	13·9	p<0·01	2·12	0·34	25·3	p<0·01
α_1 globulin	0·25	0·09	0·35	0·11	4·6	p<0·01	0·38	0·12	6·5	p<0·01	0·48	0·11	13·5	p<0·01
α_2 globulin	0·51	0·13	0·78	0·18	8·0	p<0·01	0·82	0·15	12·7	p<0·01	0·89	0·16	15·3	p<0·01
β globulin	0·73	0·18	0·93	0·15	5·0	p<0·01	0·98	0·16	7·7	p<0·01	1·07	0·17	11·7	p<0·01
γ globulin	2·03	0·34	2·03	0·44	0·0	—	2·02	0·49	0·1	0·8<p<0·9	1·79	0·45	3·6	p<0·01

[1]Test of the significance of difference from value for non-pregnant women.
[2]Blood collected within 12 hours after delivery.

TABLE II

SERUM PROTEINS OF MATERNAL AND CORD BLOOD

	African Mothers (67)		*African Infants (71)*		*European Mothers (12)*		*European Infants (19)*	
	Mean	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*
Total proteins	6·35	0·57	6·10	0·59	5·26	0·44	5·53	0·42
Albumin	2·12	0·34	2·82	0·35	2·02	0·29	2·79	0·50
α_1 globulin	0·48	0·11	0·29	0·17	0·44	0·13	0·30	0·11
α_2 globulin	0·89	0·16	0·58	0·16	0·84	0·15	0·54	0·13
β globulin	1·07	0·17	0·76	0·23	1·06	0·14	0·69	0·25
γ globulin	1·79	0·45	1·65	0·28	0·90	0·22	1·21	0·26

TABLE III

STATISTICAL SIGNIFICANCE OF RESULTS SHOWN IN TABLE II

		African Mothers Compared with European Mothers	*African Infants Compared with European Infants*	*African Infants Compared with African Mothers*	*European Infants Compared with European Mothers*
Total proteins	t	5·82	2·63	2·83	2·28
	p	p<0·01	p<0·01	p<0·01	0·02<p<0·05
Albumin		0·96	0·30	12·06	4·84
	p	0·3<p<0·4	0·7<p<0·8	p<0·01	p<0·01
α_1 globulin	t	0·55	0·36	6·97	3·17
	p	0·5<p<0·6	0·7<p<0·8	p<0·01	p<0·01
α_2 globulin	t	1·02	0·98	11·46	5·86
	p	0·3<p<0·4	0·3<p<0·4	p<0·01	p<0·01
β globulin	t	0·19	1·15	9·01	4·67
	p	0·8<p<0·9	0·2<p<0·3	p<0·01	p<0·01
γ globulin	t	6·68	6·10	2·22	3·42
	p	p<0·001	p<0·01	0·02<p<0·05	p<0·01

Buxton, 1949; Norton, Kunz, and Pratt, 1953; Sohar, Bossak, Wang, and Adlersberg, 1956; Sternberg, Dagenais-Perusse, and Dreyfuss, 1956). No significant differences were found in the mean values for albumin, α_1, α_2, and β globulins of African mothers as compared with European mothers or of African children at birth as compared with European children at birth. No correlation exists between the concentrations of these fractions in the umbilical cord blood and in the blood of the mothers. In both groups albumin concentration in cord blood is greater than in mother's blood while the concentrations of α_1, α_2, and β globulins are significantly lower in cord blood than in mother's blood. As expected the γ globulin concentration in the blood of African mothers is significantly higher than the concentration of the same fraction in the blood of European mothers. The mean concentration of γ globulin in the umbilical cord blood of European babies is significantly higher than the concentration in the blood of their mothers: good correlation exists between the concentration of the γ fraction in the cord blood and the concentration in the mother's blood (Fig. 6). The relation of the γ globulin concentration in the cord blood of African babies to the level in their mothers is complex. Examination

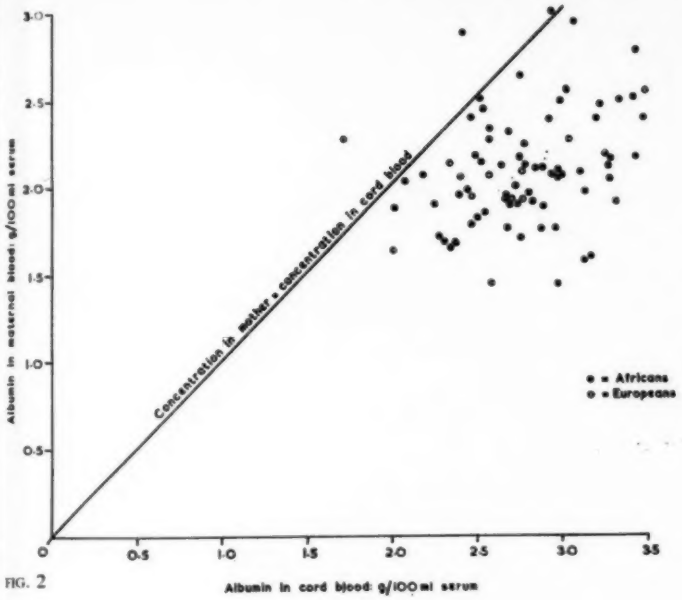

Albumin in maternal blood: g/100 ml serum
Concentration in mother = concentration in cord blood
• = Africans
o = Europeans
Albumin in cord blood: g/100 ml serum

FIG. 2

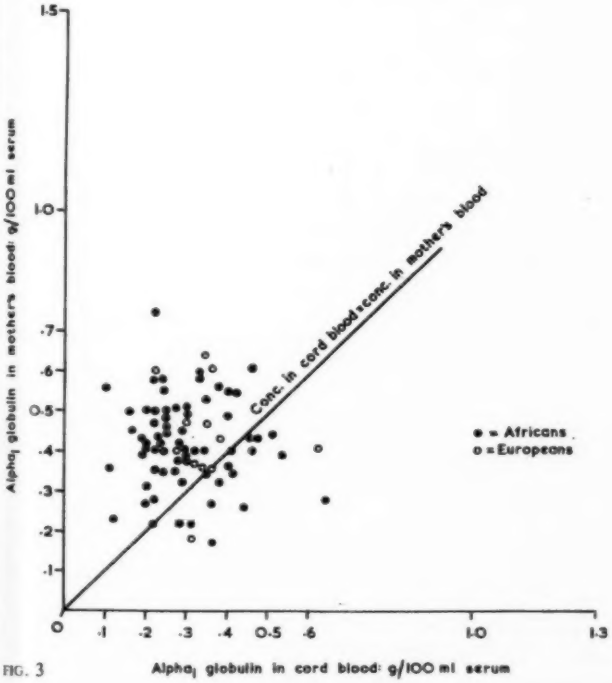

Alpha$_1$ globulin in mother's blood: g/100 ml serum
Conc. in cord blood = conc. in mother's blood
• = Africans
o = Europeans
Alpha$_1$ globulin in cord blood: g/100 ml serum

FIG. 3

FIG. 2. *Relation of albumin concentration in maternal and cord blood sera.*

FIG. 3. *Relation of* α_1 *globulin concentration in maternal and cord blood sera.*

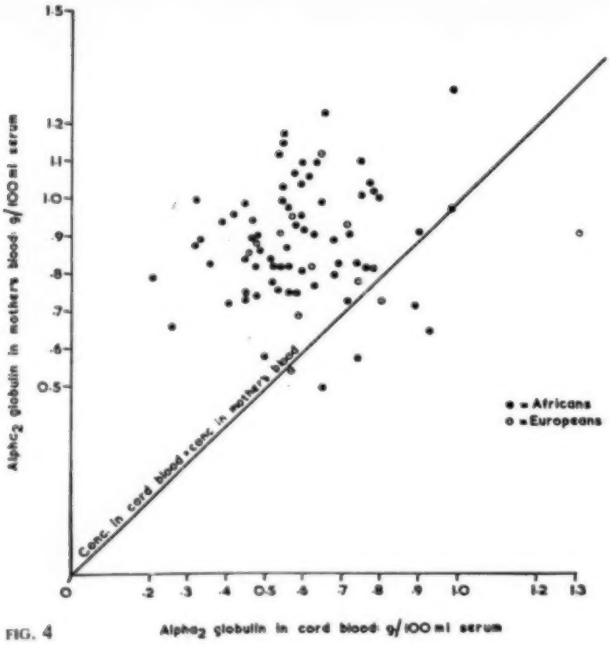

Alpha$_2$ globulin in mothers blood g/100 ml serum
Alpha$_2$ globulin in cord blood g/100 ml serum
Conc. in cord blood = conc. in mothers blood
● = Africans
○ = Europeans

FIG. 4

FIG. 4. *Relation of a_2 globulin concentration in maternal and cord blood sera.*

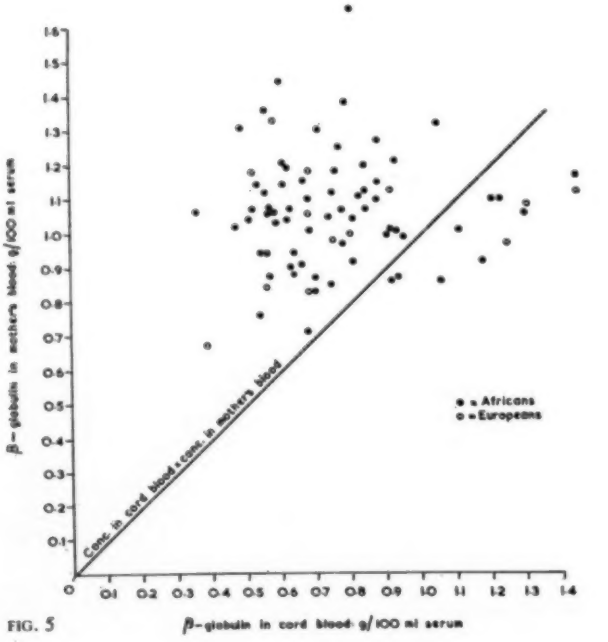

β-globulin in mothers blood g/100 ml serum
β-globulin in cord blood g/100 ml serum
Conc. in cord blood = conc. in mothers blood
● = Africans
○ = Europeans

FIG. 5

FIG. 5. *Relation of β globulin concentration in maternal and cord blood sera.*

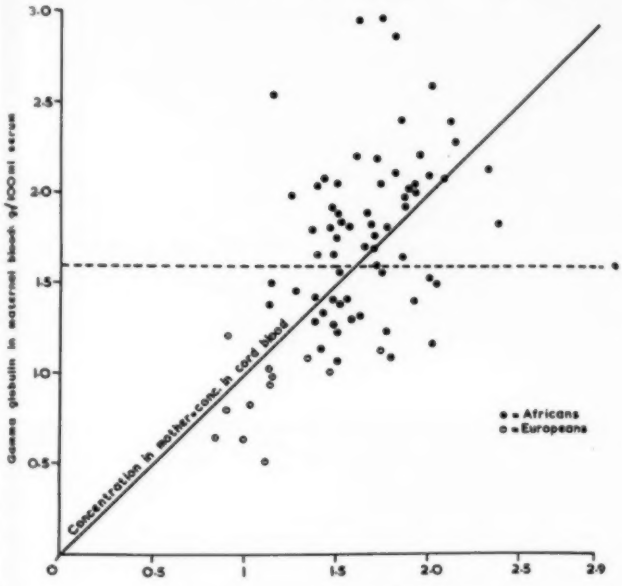
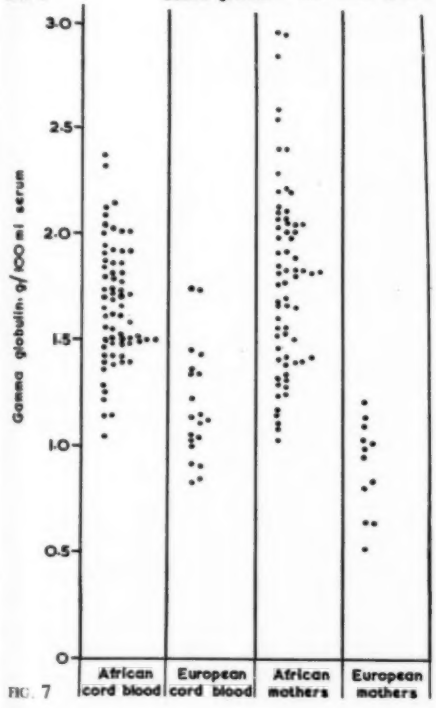

Gamma globulin in maternal blood: g/100 ml serum
Concentration in mother=conc. in cord blood
● = Africans
○ = Europeans
Gamma globulin in cord blood: g/100 ml serum

FIG. 6. *Relation of γ globulin concentration in maternal and cord blood sera.*

FIG. 7. *Gamma globulin concentrations in maternal and cord blood sera of Africans and Europeans.*



of Fig. 6 shows that African mothers with γ globulin concentrations below 1·6 g./100 ml. generally have babies whose γ globulin level is higher than that of their mother, while women with γ globulin level over 1·6 g./100 ml. have babies whose γ globulin concentration is below that of their mother. The mean γ globulin level in African umbilical cord blood is very significantly higher than the mean level in European umbilical cord blood so that the difference between the two races in this respect exists even at birth.

Fig. 8 presents two typical immuno-electrophoretic patterns in cord blood and in mother's blood. No differences have been observed between the pattern in Europeans and Africans. In most cases the cord blood shows fewer antigenic components in the α_2, β, and γ globulin zones than in the mother's blood.

In Table IV and in Fig. 9 are shown the mean values for total serum proteins and the protein fractions in healthy adults in cord blood and in children at different ages up to 6 years. At birth the total proteins are slightly lower than adult mean value, but attains this value at 6 months and stays there. The albumin level is slightly lower than adult mean value at birth but also rapidly attains that value (at 1 month). The slight drop in total serum proteins and albumin at about 2 years is probably related to the decrease in protein intake

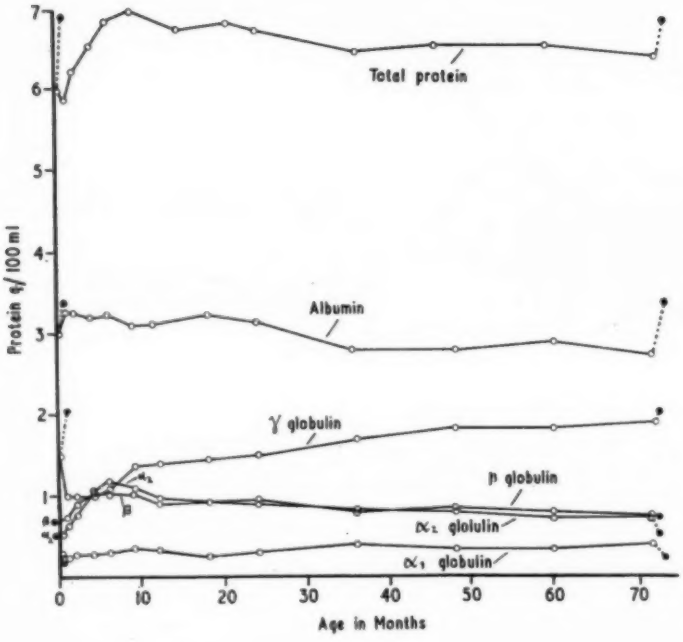


FIG. 8. *Changes in serum proteins with age (solid dots represent adult values).*

from breast milk following weaning. Alpha 1 globulin is normal at birth and does not show any notable changes throughout infancy and childhood. Alpha 2 globulin is normal at birth, rises to a maximum at about 6 months, and then falls gradually but at 6 years it has not returned to normal adult level. Beta globulin, also normal at birth, rises to a maximum at approximately 6 months and gradually returns to normal level which it reaches by the age of 3 years. At birth γ globulin concentration is about 75% of adult mean concentration: it falls rapidly to reach a minimum value by the end of the first month, remains at this low level for four to five months and begins to increase by the sixth month. A large increase occurs at 6 to 9 months of age followed by a more gradual rise and by 6 years it has not attained adult level. The rapid increase at 6 to 9 months of age is probably unrelated to malaria since the effect of malaria becomes evident after the age of 12 months (Edozien *et al.*, 1960; McGregor and

TABLE IV

SERUM PROTEINS AT DIFFERENT AGES UP TO 6 YEARS

		Adult[1] *Value*	*Cord Blood*	*1 Month*	*2 Months*	*4 Months*	*6 Months*	*8½-9½ Months*	*11½-12½ Months*	*17½-18½ Months*	*23½-24½ Months*	*2½-3½ Years*	*3½-5½ Years*	*4½-5½ Years*	*5½-6½ Years*
			(167)	(23)	(24)	(18)	(13)	(13)	(12)	(12)	(13)	(44)	(28)	(9)	(6)
Total protein	Mean	6·87	6·10	5·88	6·23	6·55	6·84	6·97	6·74	6·84	6·77	6·50	6·62	6·60	6·43
	S.D.	0·40	0·59	0·58	0·54	0·51	0·48	0·74	0·62	0·41	0·48	0·71	0·76	0·56	0·77
Albumin	Mean	3·40	2·82	3·28	3·26	3·20	3·24	3·12	3·13	3·25	3·16	2·79	2·78	2·93	2·72
	S.D.	0·31	0·35	0·46	0·43	0·39	0·38	0·41	0·37	0·33	0·40	0·52	0·49	0·51	0·47
α_1 globulin	Mean	0·23	0·29	0·23	0·28	0·28	0·30	0·35	0·34	0·25	0·29	0·39	0·34	0·34	0·40
	S.D.	0·08	0·17	0·08	0·11	0·09	0·12	0·11	0·12	0·12	0·13	0·12	0·12	0·14	0·14
α_2 globulin	Mean	0·51	0·58	0·64	0·78	1·05	1·17	1·11	0·97	0·94	0·90	0·83	0·81	0·73	0·72
	S.D.	0·13	0·16	0·18	0·19	0·23	0·28	0·32	0·18	0·18	0·21	0·17	0·16	0·14	0·13
β globulin	Mean	0·68	0·76	0·76	0·91	1·02	1·04	1·02	0·91	0·94	0·94	0·81	0·86	0·77	0·72
	S.D.	0·18	0·23	0·24	0·27	0·24	0·16	0·22	0·18	0·12	0·18	0·19	0·17	0·19	0·11
γ globulin	Mean	2·05	1·65	1·00	1·00	1·00	1·09	1·37	1·39	1·46	1·48	1·68	1·83	1·83	1·87
	S.D.	0·35	0·28	0·29	0·31	0·31	0·32	0·25	0·36	0·36	0·33	0·35	0·45	0·52	0·53

[1]Values taken from Edozien *et al.* (1960).

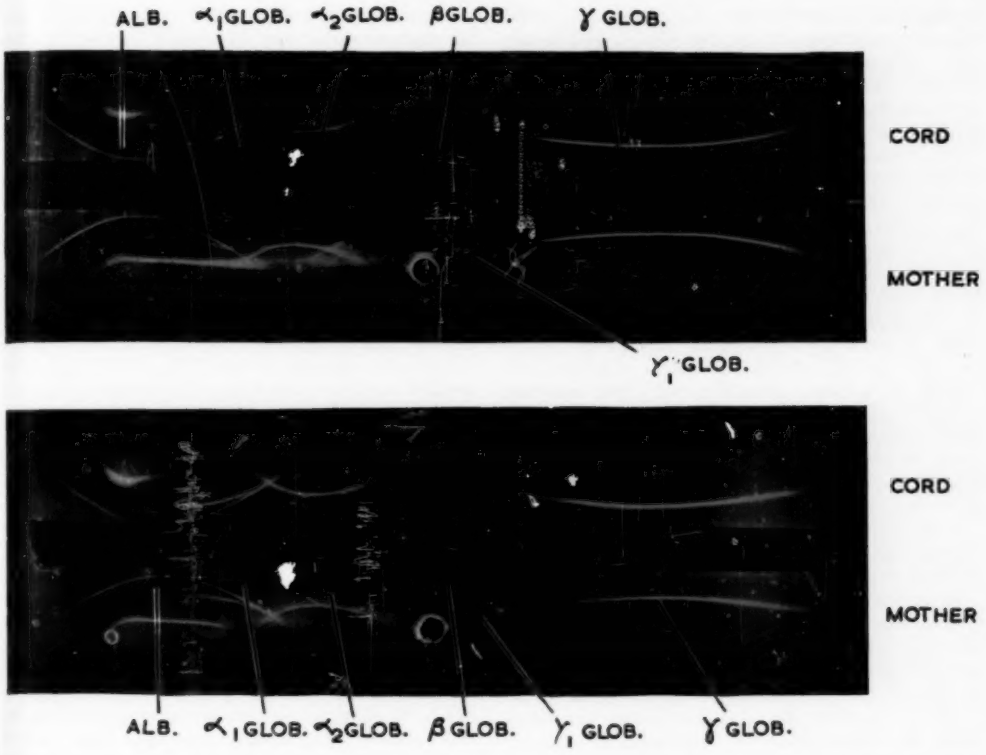


FIG. 9. *Immunoelectrophoresis of maternal and cord blood sera (horse-immune serum from Pasteur institute, Paris, was used). Note that maternal sera have at least two components each in the α_2 and β globulin envelopes while the cord blood sera show only one component each distinctly. Also the γ_1 globulin zone is not well developed in cord blood sera.*

Gilles, 1959). In general these changes follow the pattern described in Europeans (Longsworth *et al.*, 1945; Moore *et al.*, 1949; Sternberg *et al.*, 1956; Homolka and Mydlil, 1955; Oberman, Gregory, Burke, Ross, and Rice, 1956; Josephson and Gyllensward, 1957) except that in Africans the changes in γ globulin occur at a higher level.

DISCUSSION

These data show very clearly that the difference in serum γ globulin concentration between Africans and Europeans is present at birth. The question which arises from this observation is whether the concentration of γ globulin in the serum of the newborn is a genetically determined value or whether it is dependent on the level in the mother.

Close correlation has been demonstrated between the level of γ globulin in cord blood and in maternal blood and this indicates that conditions in the mother probably control the γ globulin content of the serum in the newborn child. The rapid decline in serum γ globulin concentration during the neonatal period to reach a steady low level by the end of the first month further suggests that before parturition there may be a transfer of γ globulin from maternal to foetal circulation since most of the γ globulin so transferred before birth would be expected to disappear from the infant's blood within 28 days, assuming that the half-life of human serum γ globulin is 10 to 20 days (London, 1950; Dixon, Maurer, and Deichmiller, 1953; Dixon, Talmage, Maurer and Deichmiller, 1952). Moreover, there is evidence that in man antibodies, which are γ globulins (Tiselius and Kabat, 1939; Williams, Petermann, Colovos, Goodloe, Oncley, and Armstrong, 1944; Enders, 1944; Haurowitz, 1947; Boyd, 1954; Isliker, 1957), can be transferred *in utero* from

mother to foetus (Wiener and Silverman, 1940; Kuttner and Ratner, 1923; Ten Broeck and Bauer, 1923; Pommerenke, 1936; Ratner, Jackson, and Gruehl, 1927; Sherman, Hampton, and Cooke, 1940; Bell and Eriksson, 1931; Neill, Gaspari, Richardson, and Sugg, 1932; Wright and Clerk, 1944). Indeed several observers (Moore *et al.*, 1949; Good and Zak, 1956; Franklin and Kunkel, 1958) believe that the newborn infant is unable to synthesize γ globulin and that therefore all the globulin present in the foetus at birth is derived from the mother by transfer through the placenta. This conclusion is based upon the following evidence: (1) After birth the serum γ globulin level gradually falls until the onset of antibody synthesis at the age of 5 to 6 months. (2) Only specific antibodies present in the mother have been found in cord blood and the amount in cord blood is usually below the level in maternal blood. (3) The higher molecular weight (19S) fraction of the globulin is absent from cord blood. (4) The observation of Good and Zak (1956) that the baby of an agammaglobulinaemic mother was also agammaglobulinaemic at birth and during the neonatal period and did not begin to form γ globulin until the end of the second month of life.

However, uterine transmission is probably not a simple exchange of serum proteins across the placenta. Cohen (1950) has observed that in the rabbit, which has a placenta very similar in structure to that of man, the passing of homologous serum antibody through the placenta differs markedly from the transmission of heterologous serum γ globulin. The concentration of albumin is significantly higher in cord blood than in mother's blood while the concentrations of α_1, α_2, and β globulins are significantly lower in cord blood. The immunoelectrophoretic data also demonstrate that some protein components with the electrophoretic mobility of α_2, β_1, and γ_1 globulins may be absent from cord blood although present in maternal blood. While it is possible to explain most of the differences in protein composition between maternal and foetal sera in the human species by haemoconcentration of foetal blood, coupled with the presence of large molecules within the α_2, β_1, and γ_1 globulin envelopes, nevertheless, the observation that the transfer of γ globulin from mother to foetus appears to be inhibited when the concentration of γ globulin in the maternal blood rises above a certain critical value, does suggest that in man the transfer of γ globulin from mother to foetus is a more complex process than simple diffusion across the placenta.

The concentration of γ globulin in the blood of the newborn is the result of the rapidly declining component derived from the mother prior to parturition and the rising contribution by the baby under the influence of its genetic constitution and/or in response to environmental stimuli. Much further investigation is needed before it is possible to assess clearly the relative importance of each of these factors.

During infancy and childhood the changes in the serum γ globulin concentration in Africans follow the pattern which has been described in Europeans but at a higher level. Here also the questions may be asked whether the African has inherited a greater innate capacity to form γ globulin or whether his high γ globulin level is an expression of a more highly infected environment or whether his normal environment contains infective agents which provoke greater γ globulin response? Attemps have been made to answer these questions by a study of the concentration of γ globulin in the serum of Africans living under European conditions of life (Schofield, 1957; Van Oye and Charles, 1957). This approach is not likely to produce valid and conclusive evidence, because the capacity of individuals to respond to any situation should normally be assessed after applying the correct stimulus; hence, in order to answer these questions, it would be more appropriate to study the γ globulin concentration in Europeans and in other races under conditions comparable to those in which the majority of Africans grow up. It is not sufficient only to investigate Africans who have never been exposed to the necessary environmental stimuli. There are a number of natural situations in the world at present where other races can be investigated under African conditions of life and valuable information may accrue from such a study. Antibodies probably constitute the principal basis of immunity, and because antibodies are γ globulins and there is little doubt that individuals and ethnic groups may vary in inborn resistance to endemic infections, further study of this problem may enhance our understanding of race and group immunity.

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A stable sheep cell preparation for detecting thyroglobulin auto-antibodies and its clinical applications

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SYNOPSIS The preparation of stable formolized sheep cells coated with thyroglobulin and their use for the detection of auto-antibodies in human thyroiditis is described. This provides an easy laboratory test which gives results closely comparable with those using fresh tanned cells.

The agglutination titres do not closely parallel the antibody content of the serum but a good correlation may be obtained by inhibiting agglutination with thyroglobulin.

The diagnostic usefulness of the test in treating disorders of the thyroid is discussed and related to the results of tests for the other two immune systems involved in thyroiditis. The tanned red cell test may be used to differentiate Hashimoto's disease from thyroid cancer and non-toxic colloid goitre, and to assess the choice of treatment in patients with Graves' disease. It has proved of particular value in genetic studies of auto-immunity.

The detection of antibody by agglutination of tannic-acid-treated red cells coated with antigen (Boyden, 1951) was first applied to the study of thyroid auto-immunity in animals by Witebsky and Rose (1956); it was shown that the serum of rabbits immunized with autologous or homologous thyroid extracts would agglutinate tanned cells coated with the thyroid antigen, often to a high titre. Following the demonstration of thyroid auto-antibodies in the serum of patients with Hashimoto's thyroiditis a number of studies have been reported in which the tanned cell method was applied to the detection of auto-antibodies in human thyroid diseases (Witebsky, Rose, Terplan, Paine, and Egan, 1957; Paine, Terplan, Rose, Witebsky, and Egan, 1957; Roitt and Doniach, 1958; Owen and Smart, 1958; Blizzard, Hamwi, Skillman, and Wheeler, 1959; Cline, Selenkow, and Brooke, 1959). The antigen responsible for the agglutination of tanned coated cells with thyroiditis sera was shown to be thyroglobulin which also gives rise to the precipitin reaction in agar gel. The tanned red cell agglutination test is of the order of 1,000 times more sensitive than the precipitin reaction, and although a positive result of low titre is not diagnostic of Hashimoto's disease, the test has proved interesting and useful in the investigation of all types of thyroid disease and theoretical problems associated with auto-immunity.

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The preparation of fresh tanned coated cells is time-consuming and therefore inconvenient for use in the routine laboratory. This difficulty has now been overcome by treating these cells with formalin to give preparations which agglutinate specifically yet are stable for many months on storage at 4°C. Fulthorpe (1957) previously used this technique successfully for preserving cells coated with tetanus toxoid.

PREPARATION OF FORMOLIZED THYROGLOBULIN-COATED SHEEP CELLS

REAGENTS The following were made up:—

1 *Phosphate buffer pH 7·2* Anhyd. KH_2PO_4, 12 anhyd. Na_2HPO_4, 40·4 g., and NaCl, 36·0 g. are dissol in distilled water and made up to 5 litres.

2 *Borate-succinate buffer, 0·05M, pH 7·5* Adc cinic acid solution, 5·9 g./litre ($Na_2B_4O_7$ 10H 0; litre) to 3 litres of borax solution until the *p*H is 7·- Nearly 3 litres is required for this. NaCl is added to 0·75%. Add normal horse serum which has previously been heated to 56°C. for 30 min. to a final concentration of 1%.

3 *Tannic acid solution, 1 : 20,000* Tannic acid, 12·5 mg (Hopkin & Williams or Merck reagent grade products were used) dissolved in 250 ml. phosphate buffer *p*H 7·2 is freshly prepared before use.

4 *Human thyroglobulin solution* Freeze-dried thyroglobulin (prepared by the method of Derrien, Michel,

Roche, 1948), 0·5 g., is dissolved in 250 ml. phosphate buffer *p*H 7·2.

Formalin (40% formaldehyde).

PREPARATION OF PRESERVED TANNED CELLS

1 Sufficient fresh sheep cells to give a packed cell volume of 10 ml. are washed three times with physiological saline and made up to 250 ml. in phosphate buffer *p*H 7·2.

2 The cell suspension and 250 ml. of tannic acid solution are warmed separately to 37° C. and then combined and incubated in a water bath at 37°C. with occasional mixing for 15 minutes.

3 The tanned cells are gently centrifuged (six minutes at 1,000 r.p.m.) and suspended in 500 ml. of phosphate buffer. The suspension is divided into two equal portions (ultimately to become the test and control cells) and the tanned cells are spun down.

4 The first batch of cells is re-suspended in 250 ml. phosphate buffer and 250 ml. of human thyroglobulin solution is added. The mixture is incubated with occasional mixing for 30 minutes at 37°C.

5 The coated cells are gently centrifuged, washed twice with phosphate buffer, and suspended in 500 ml. of the borate-succinate buffer *p*H 7·5.

The second portion of cells is suspended in borate-succinate buffer without coating and used for absorption of the sera before the test and as control cells in the test.

6 Both cell suspensions are stirred mechanically and 50 ml. of formalin is added to each batch slowly over a period of 20 to 30 min. (slow addition avoids haemolysis).

7 After standing for 18 hr. at 4°C., a further 50 ml. of formalin is added.

8 When the cells have settled and adhere to the bottom of the container, *i.e.*, after one to three days, the supernatant is poured off and is replaced with fresh borate-succinate buffer; the cells are re-suspended by vigorous shaking. They are again allowed to settle and washing is repeated once more.

9 Finally both batches of cells are diluted to give a 1% suspension in borate-succinate buffer; 0·2% formalin is added to the final 1% suspension as a preservative. The cells are stored at 2 to 5°C. and must be shaken thoroughly before use to provide a uniform suspen-

AGGLUTINATION TEST FOR THYROGLOBULIN AUTO-ANTIBODIES

Heterophile antibodies are first absorbed using control coated cells: 0·1 ml. of patient's serum (or 3 drops delivered with a '30-dropper pipette') is added to 0·4 ml. of the control cell suspension. After 10 minutes the cells are spun down and the supernatant (now a 1 in 5 dilution of serum) is serially diluted in saline. For routine testing it is convenient to make serum dilutions of 1 in 5, 1 in 25, and then 10-fold dilutions to 1 in 2·5 million with a single pipette. This procedure leads to considerable errors at high dilutions but is satisfactory for diagnostic tests; if accurate end-points are required a separate pipette must be used for each dilution.

To 0·1 ml. of each serum dilution is added 0·1 ml. of the thyroglobulin-coated test cells while 0·1 ml. of uncoated cells is added to 0·1 ml. of 1 in 5 serum dilution as a control. The cells and serum are gently mixed and allowed to stand undisturbed at room temperature for three hours or overnight before reading the results. If a batch of sera is tested together it has been found convenient to set up dilutions up to 2,500 only in the first place, leaving the next dilution without cells for one hour or until the test can just be read. If positive at 1 in 2,500 the series of dilutions is completed and cells added to them. This saves coated cells and technicians' time, particularly where the test is done routinely on all goitre patients. The test is extremely sensitive and it is essential to use a clean pipette for each serum if false weak positive results are to be avoided. The curvature at the base of the tubes may be a critical factor in affecting the stability of a positive agglutination pattern.

The most consistent results are obtained by reading the end-point as the highest dilution at which the cells settle to form a diffuse carpet over the bottom of the tube or cup. A ± result appears as a smaller circle of cells having a dark outer rim, while a negative test shows as a tight button of cells (Fig. 1). The ± results are ignored. The control cup should always be negative; if agglutination is observed the 1 in 5 serum dilution should be further absorbed using packed control cells. The dilutions and

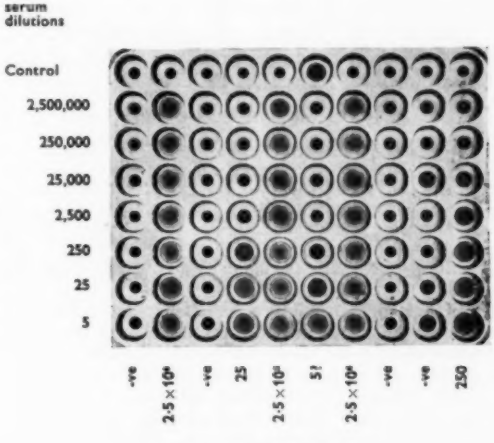

FIG. 1. *Direct haemagglutination test on 10 sera in Perspex tray. Each individual serum is progressively diluted in seven cups of a vertical row, the eighth cup being set up with the 1 in 5 serum dilution and control uncoated cells. Agglutination leads to a diffuse mat of cells whereas negative sera produce a tight button of cells at the bottom of the cup. Intermediate results (±) are regarded as negative. One of the sera shows incomplete absorption of heterophile antibodies as evidenced by a positive reaction in the control cup.*

subsequent tests are most readily carried out in Perspex agglutination trays containing 10 × 8-1 ml. cups.[1] For cleaning, the trays and pipettes are rinsed in running water and immersed in hypochlorite solution overnight (100 ml. of sodium hypochlorite, 15% available chlorine, to 1 gallon of water). They are then rinsed in hot tap water followed by distilled water. The trays are dried in air at room temperature and the pipettes in a hot drying oven.

COMPARISON OF RESULTS OBTAINED WITH FRESHLY PREPARED TANNED CELLS AND PRESERVED FORMOLIZED CELLS

Sera previously tested against freshly prepared thyroglobulin-coated cells were retitrated with a preserved cell preparation. In 88 out of 107 sera, identical results were obtained with both preparations; the preserved cells gave a titre two tubes lower with one serum, one tube lower with 13 sera, one tube higher with four sera, and two tubes higher with one serum. In view of the fact that serial dilutions with a single pipette were made on separate occasions and that ± agglutination patterns were excluded when reading the end-point, these results show that titres obtained with the preserved cells are comparable with those previously reported in the literature using fresh unformolized cells. Different batches of preserved formolized cells gave identical results even after nine months' storage at 4°C.

RELATIONSHIP OF TANNED RED CELL TITRE TO ANTIBODY LEVEL

The direct agglutination titre was determined on 20 sera diluted volumetrically and compared with antibody estimations made by two other methods.

1 CO-PRECIPITATION WITH RADIO-ACTIVE ANTIGEN An excess of I^{131} thyroglobulin was mixed with the serum and a rabbit anti-human-γ-globulin serum added to precipitate the immune complexes (Roitt and Doniach, 1959). The radio-activity of the washed precipitate provided a measure of the antibody content of the original serum, the method being calibrated against sera having known antibody concentrations determined by the Heidelberger and Kendall (1935) quantitative precipitin technique.

2 HAEMAGGLUTINATION INHIBITION TEST WITH THYROGLOBULIN-SENSITIZED CELLS Quantities each of 0·5 ml. of a thyroglobulin solution (2 μg./ml.) were dispensed in a series of ¾ in. diameter tubes, and 0·5 ml. of suitable serum dilutions added, mixed by inversion, and stood at room temperature for one hour; 0·1 ml. of sensitized cells was added to each tube and mixed as before. The end-point was read the following day. After preliminary titration using 0·5 ml. of 10-fold dilutions of serum, the test was repeated using two-fold dilutions over the appropriate range, *i.e.*, between the last tube showing full agglutination and the first tube showing a clear negative. Further accuracy can be obtained using serum dilutions differing by 20%. The amount of antibody contained in the calculated volume of undiluted serum which would give clear-cut agglutination when mixed with 1 μg. of thyroglobulin is defined as 1 milliunit of anti-thyroglobulin. The test is always carried out at a constant thyroglobulin level since it has been found that there is no linear relationship between the inhibiting dose of antigen and the dilution of a given serum. This is illustrated in the Table. The phenomenon has also been observed in haemagglutination inhibition tests using tetanus toxin-antitoxin mixtures (Fulthorpe, 1958).

[1]Obtained from R. B. Turner & Co. Ltd., 102, Church Lane, London, N.2.

TABLE

HAEMAGGLUTINATION INHIBITING CAPACITY OF THYROGLOBULIN FOR HASHIMOTO SERUM AT DIFFERENT CONCENTRATIONS OF REACTING MIXTURES

Volume of Serum Used (ml.)	*Dose of Thyroglobulin Required for Inhibition (μg.)*
0·1	12·5
0·01	2·0 (1·25)[1]
0·001	0·55 (0·125)

[1]The figures in brackets indicate the expected inhibiting dose by calculation and demonstrate the great difference in inhibiting capacity of thyroglobulin at different levels of serum concentration.

The comparative results are presented in Figs. 2A and 2B. It is apparent that antibody levels determined by the radioactive coprecipitation and quantitative precipitin methods correlate far more closely with the haemagglutination inhibition values than with the direct tanned red cell agglutination titre.

CLINICAL APPLICATIONS OF THE TANNED RED CELL AGGLUTINATION TEST

In clinical practice thyroid antibody tests are of help in the differentiation of Hashimoto's disease (lymphadenoid goitre) from thyroid cancer and non-toxic colloid goitres, in the diagnosis of primary myxoedema, and in the assessment of the degree of lymphadenoid changes in Graves' disease. The tests are of interest in the follow-up of patients with thyroiditis and may eventually lead to a better understanding of the pathogenesis of the disease and help to elucidate its connexions with other diseases of auto-immune aetiology.

The diagnostic usefulness and clinical applications of the tanned cell test cannot easily be discussed without reference to the other auto-antibodies present in patients with thyroid diseases, either

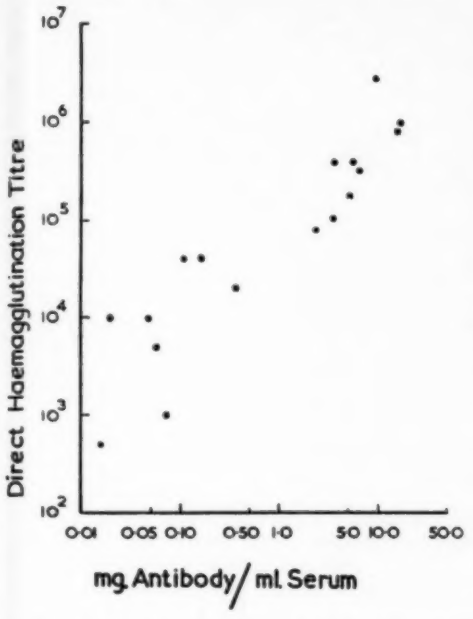

FIG. 2A. *Comparison of direct agglutination titres with antibody levels determined by quantitative techniques showing rather poor correlation in these two tests.*

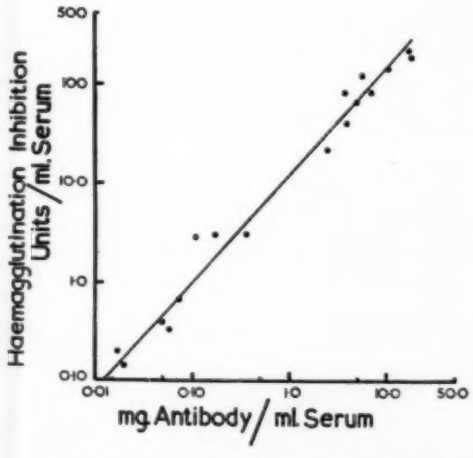

FIG. 2B. *A better correlation is obtained when the quantitative antibody estimation is plotted logarithmically against the result of the haemagglutination inhibition test. The position of the line was calculated by the method of least squares.*

separately or in combination with anti-thyroglobulin. The most studied of these is the complement-fixing antibody against the microsomal antigen of thyroid epithelial cells (Trotter, Belyavin, and Waddams, 1957; Roitt and Doniach, 1958; Belyavin and Trotter, 1959; Anderson, Goudie and Gray, 1959). The complement-fixation test using thyrotoxic gland extracts as antigen is best carried out concurrently with the tanned red cell agglutination test for routine purposes; when the two tests are combined, one experienced technician can comfortably examine 40 sera in one day. The tanned red cell agglutination test is put up in dilutions as described above, but for the complement-fixation test the sera are first screened at 1 in 4 and 1 in 20 dilutions, in similar perspex trays. All positives are then put up again in serial dilutions from 1 in 4 to 1 in 512 against thyrotoxic thyroid and human liver to determine whether the antibodies are specific for the thyroid gland. Sera giving tanned red cell agglutination of over 2,500 are also set up in Ouchterlony gel plates for the precipitin test. Using these three tests, over 90% of Hashimoto patients and 80% of primary myxoedema cases are found to have thyroid antibodies (cf. Smart and Owen, 1960). However, owing to the extreme sensitivity of the tanned red cell agglutination test, positive results of low titre are also obtained in patients with other thyroid disorders who have a non-progressive focal thyroiditis of no clinical significance.

DIFFERENTIATION OF THYROIDITIS FROM CANCER

About 30% of patients with thyroid cancer have a positive tanned red cell or complement-fixation test so that the serological reactions can only be used in conjunction with careful clinical assessment and with other tests of thyroid function. Fortunately the majority of Hashimoto patients have high titre antibodies both against thyroglobulin and the microsomal antigen whereas cancer patients give low titres and are only positive in one or other test as a rule. Fig. 3 shows the distribution of antibody titres in 303 cases of lymphadenoid goitre and in 64 cases of thyroid malignancy. Eighty-four per cent. of Hashimoto patients had tanned red cell titres of 25,000 or more with positive precipitins in two-thirds of cases, and complement-fixation titres of 64 or higher, whereas only 4·7% of cancer cases had comparable titres and only one patient showed precipitins.

The problem is more difficult when thyroiditis patients have low-titre antibodies which are of no diagnostic help. Such cases frequently have raised γ-globulins and this probably reflects the presence of other antibodies not detected by the tanned red cell or complement-fixation tests. It is therefore particularly useful to do the sero-flocculation

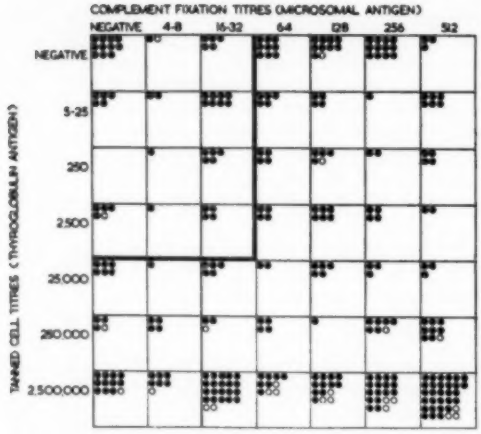

FIG. 3A. *Incidence of thyroglobulin and microsomal antibodies in Hashimoto's thyroiditis. Each patient is represented by an open (male) or solid (female) circle and placed according to the serum titre obtained in each of the two tests. Eighty-four per cent. of patients had tanned red cell titre of 25,000 or over and complement-fixation titres of 64 or greater, i.e., beyond the thickened line. The majority of patients with high tanned red cell titres also gave positive precipitins in agar gel. All the 11 patients negative by the tanned red cell and complement-fixation tests were subsequently shown to have antibodies to the second antigen of the thyroid colloid (CA2) demonstrated by the fluorescent antibody technique.*

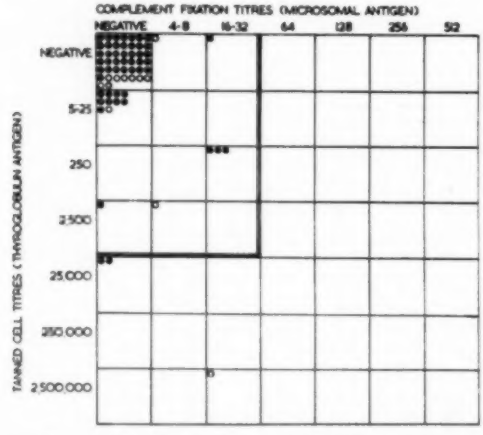

FIG. 3B. *Incidence of the same two thyroid auto-antibodies in cases of thyroid cancer. Only one case had a positive precipitin test. The fluorescent antibody technique showed weak antibodies to CA2 in only three of the cases negative by the tanned red cell and complement-fixation tests.*

reactions, especially the thymol and zinc sulphate turbidity tests. There is now concrete evidence of the existence of thyroid antibodies not detected by the tanned red cell or complement-fixation tests. Hashimoto patients with negative results in these two tests (cf. Doniach, Hudson, and Roitt, 1960) have antibodies against a second antigen of the thyroid colloid (CA2) distinct from thyroglobulin, which can only be demonstrated by Coons' fluorescent antibody technique (Balfour, Doniach, Roitt, and Couchman, 1961). The fluorescent test is also the most sensitive and specific for the microsomal (CF) antibodies which give cytoplasmic staining in frozen unfixed thyroid sections. Although the fluorescent antibody technique is perhaps too complicated and laborious to be used in routine laboratories, most Hashimoto cases can now be diagnosed by the three simpler tests since even low titre antibodies may be significant if tanned red cell and complement-fixation tests are positive together or if the patient has raised flocculation values.

DIFFERENTIATION OF THYROIDITIS FROM NON-TOXIC-NODULAR GOITRES With regard to distinguishing colloid non-toxic goitres from Hashimoto's disease in the absence of impaired thyroid function the tanned cell test is not diagnostic in titres under 250·000 unless precipitins are also positive. A small amount of antibody may occasionally give a high agglutination titre as can be seen in Fig. 2A. This is not necessarily associated with significant lymphoid changes in the thyroid gland. It is not known whether this implies that some patients may be immunized against thyroglobulin without having any lymphadenoid invasion of the gland. This is a possibility, since animals immunized with alum-precipitated homologous thyroglobulin usually have circulating antibodies but rarely show destructive lesions of the thyroid.

Only 3·5% of patients with colloid goitres have complement-fixing titres greater than 1 in 4. Furthermore, patients with this disease never give positive precipitins unless there is a substantial superimposed thyroiditis in the goitre.

DIAGNOSIS OF PRIMARY MYXOEDEMA Next to Hashimoto's disease, the highest antibody levels are found in primary adult myxoedema. Twenty per cent. of cases have precipitins with tanned red cell titres of over 2½ million and many more have lower titres in the tanned cell test. The complement-fixation test is often positive as well. Hospital patient surveys show an incidence of 10% (Hill, 1961) up to 30% (Hackett, Beech, and Forbes, 1960; Mackay and Perry, 1960) of positive tanned red cell tests (depending upon the inclusion of patients with connective tissue

diseases) in middle-aged women without overt thyroid disease so that the diagnostic index of the agglutination test is poor for myxoedema unless other antibodies are also present or support is obtained for the diagnosis from a low protein-bound iodine level or basal metabolic rate. However, in some cases of incipient hypothyroidism, high titre antibodies are present while the usual thyroid function tests are still boderline.

AUTO-IMMUNE THYROIDITIS ASSOCIATED WITH THYROTOXICOSIS Thyroid antibodies are found in about two-thirds of cases of primary thyrotoxicosis, whether untreated or treated with antithyroid drugs. The great majority of patients have tanned red cell titres of 25 to 2,500 and/or complement-fixation test titre of 1 in 4 to 1 in 32. In about 10% of thyrotoxics, especially those with severe eye signs, antibody titres are in the Hashimoto range (Hales, Myhill, Rundle, Mackay, and Perry, 1961; Doniach and Roitt, 1961). These high antibody titres reflect fairly extensive lymphadenoid lesions and such patients frequently become myxoedematous after subtotal thyroidectomy or after radio-iodine treatment. If they are given antithyroid drugs, these patients often require small maintenance doses and eventually go into prolonged remissions of their hyperthyroid symptoms. If the thyroiditis process is intense and progressive, myxoedema may supervene several years after the thyrotoxic episode, but most patients with high antibody titres probably remain euthyroid for many years (10-20). A low-grade auto-immune thyroiditis is compatible with repeated recurrences of toxicity and in such cases the antibody titres remain low compared with those of progressive thyroiditis in which antibody levels show a steady rise during the period of lymphoid replacement in the gland.

Early Hashimoto's disease may sometimes simulate mild thyrotoxicosis in that patients complain of nervousness and palpitations. These cases can be distinguished from true Graves' disease by the fact that their I^{131} uptake can be suppressed with triiodothyronine (120 μg. daily for 10 days) and also because they fail to improve when given antithyroid drugs. The tanned red cell test may be completely negative and only complement-fixing antibodies, sometimes to high titres, and anti-CA2 are present. It may be that acinar destruction due to as yet unknown factors releases metabolically active iodinated proteins which remain in the circulation in the absence of antibodies to remove and inactivate them (Roitt and Doniach, 1960).

EFFECT OF TREATMENT ON ANTIBODY LEVELS After operation for Hashimoto goitre, the precipitin level decreases sharply but the tanned red cell test remains positive for many years after thyroidectomy owing to its great sensitivity. In Hashimoto patients treated with thyroid hormone, precipitins remain positive for some years and the tanned red cell test remains of high titre even after the goitre has gone down completely. Complement-fixation titres usually decrease in treated patients.

In thyrotoxic patients with focal thyroiditis, I^{131} treatment may lead to an increase in the complement-fixation antibody level, possibly as a result of increased antigen release, though the tanned red cell titre appears to remain unchanged. In well-established primary myxoedema, antibody levels do not change with replacement therapy (Owen and Smart, 1958); this does not necessarily apply to the early phase of atrophic thyroiditis.

To summarize, it is safest to rely on the precipitin test when Hashimoto's disease has to be distinguished from thyroid cancer, but even this requires the support of radioiodine topographical studies as well as careful clinical assessment of each individual case. A positive precipitin test indicates widespread thyroiditis even in patients with underlying thyrotoxicosis but this occurs in less than 1% of cases. In patients with negative precipitins, a high tanned red cell titre, together with a high complement-fixation titre, is strongly indicative of lymphadenoid goitre, whether the patient is euthyroid or hyperthyroid. Low titres or even completely negative results in both tests do not rule out Hashimoto's disease but additional help may be obtained from the thymol and zinc sulphate turbidity tests and from the fluorescent test for antibodies to the second antigen of the thyroid colloid. Needle biopsy is advisable in difficult cases.

Thyrotoxic patients with antibodies may be treated medically for a trial period while the trend of their antibody titre is established. If titres are low and remain stationary, operation is unlikely to cause myxoedema but if the titres are found to rise progressively it is preferable to avoid surgery or radio-iodine treatment unless cardiac involvement renders a rapid cure imperative.

We are grateful to Professor F. Dickens, Professor Sir Charles Dodds, and Mr. R. Vaughan Hudson for their interest and constant support in this work, and to Professor A. Kekwick and the Clinical Research Committee for facilities afforded in the Institute of Clinical Research.

We wish to thank the pathologists, surgeons, and physicians from the Middlesex Hospital and many other hospitals who kindly sent us serum from their patients for thyroid antibody tests. Without their help we could not have gathered the material on which this work was based.

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J. clin. Path. (1961), **14**, 661

A note on the determination of serum beta-glucuronidase activity

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SYNOPSIS A modification of the method of Fishman, Springer, and Brunetti (1948) for determining serum β-glucuronidase activity is described. Normal values are presented and some of the factors which might influence the serum enzyme determination have been investigated.

The concentration of the enzyme β-glucuronidase in blood has been of interest in the study of various types of cancer and other diseases (McDonald and Odell, 1947; Cohen and Huseby, 1951; Fishman, Kasdon, Bonner, Fishman, and Homburger, 1951; Follette, Valentine, and Lawrence, 1952; Hackensellner, Seelich, and Lind, 1958; Ohkubo, Sonoda, and Kusunoki, 1958; Mattea, Pietra, and Viola, 1959; Goldbarg, Pineda, Banks, and Rutenburg, 1959; Pineda, Goldbarg, Banks, and Rutenburg, 1959; Whitaker, 1960). The methods most commonly used for the determination of the enzyme level are those of Talalay, Fishman, and Huggins (1946), where the enzyme acting upon phenolphthalein mono-β-glucuronic acid liberates phenolphthalein which is estimated colorimetrically, and of Fishman, Springer, and Brunetti (1948) using the same substrate but protein being removed by precipitation with trichloroacetic acid. While using the latter method it was noted that the final *p*H was not satisfactorily adjusted to the optimum value and that errors arose because of adsorption of phenolphthalein on the protein precipitate. The following is an account of the modifications introduced to overcome these difficulties.

MODIFIED METHODS

ADJUSTMENT OF INITIAL *p*H Fishman *et al.* (1948) used an acetate buffer, *p*H 4·5, 0·1M concentration, but it was found preferable to use twice the strength (0·2M, *p*H 4·5) to ensure that the *p*H was adequately adjusted for the incubation period.

FINAL *p*H At the end of incubation when the alkaline solution is added, the *p*H should be adjusted to between 10·2 and 10·5 for maximum stable colour development. In experiments using the alkaline reagent of Fishman *et al.* (1948), it was found that in some instances the *p*H was 9·8 (even although in these particular experiments 0·1M (*p*H 4·5) acetate buffer was used for the incubation mixture). A carbonate solution *p*H 10·5 (similar to that described by Abul-Fadl, 1957) was substituted for the alkaline reagent, and this gave a satisfactory final *p*H.

LOSS OF PHENOLPHTHALEIN BY PROTEIN PRECIPITATION In recovery experiments where known amounts of phenolphthalein were added to serum and then estimated after the method of Fishman *et al.* (1948) had been carried out, considerable loss of the dye was demonstrated (Table I).

TABLE I

RECOVERY OF ADDED PHENOLPHTHALEIN (Fishman *et al.* (1948) method)

Phenolphthalein Added to Serum (μg./ml. Final Concentration)	*% Recovery*
5	21
5	30
10	27
15	40

Experiments were made to see what errors were caused by the presence of unprecipitated protein in the final solution. Table II demonstrated that the proportion of phenolphthalein detected varied considerably according to the amount added.

TABLE II

RECOVERY OF ADDED PHENOLPHTHALEIN IN SOLUTIONS STILL CONTAINING SERUM PROTEIN

Serum	*Phenolphthalein Added (μg.)*	*% Phenolphthalein Detected in Final Protein-containing Solution*
a	1	60
a	2	55
b	4	86
c	5	96
d	6	92
c	10	95

In addition, in many sera, there were turbid final solutions and it was therefore decided that for this reason

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and because of the protein binding of the dye, non-precipitation of the protein was unsatisfactory.

The method finally evolved was to coagulate the protein by heat and, after cooling, add the carbonate solution directly to this solution. Fishman *et al.* (1948) used heat precipitation when investigating the β-glucuronidase activity in vaginal fluid but afterwards spun down the precipitate and added alkaline reagent only to the decanted supernatant solution. By adding the carbonate solution directly to the heat-coagulated solution the phenolphthalein was almost completely recovered (Table III). A small proportion of the total protein remained uncoagulated but was not sufficient to interfere with the phenolphthalein colour development.

TABLE III

RECOVERY OF ADDED PHENOLPHTHALEIN AFTER HEAT COAGULATION

μg. *Phenolphthalein Added/ml. Final Solution*	*% Recovered in Duplicate Solutions*	
2	105	100
4	102	97
6	100	101
8	100	102
10	99	101
12	96	100
14	100	100
16	98	98
18	97	98
20	100	97

KINETICS OF SERUM β-GLUCURONIDASE

To determine the most suitable conditions for estimating the enzyme, kinetic experiments were carried out with several different, unpurified sera.

*p*H OPTIMUM All serum values using phenolphthalein mono-β-glucuronic acid as substrate reported in the literature have been determined at *p*H 4·5, but when a *p*H optimum curve was attempted using the heat coagulation method, at certain *p*H levels there was no or incomplete precipitation and the final solutions were turbid. Table IV indicates the errors which arose by coagulation at different *p*H levels.

TABLE IV

RECOVERY OF ADDED PHENOLPHTHALEIN AFTER COAGULATION OF SERUM PROTEIN AT DIFFERENT *p*H LEVELS

*p*H	*% Recovery of Added Phenolphthalein (Mean of Triplicate Values)*
3·8	86
4·2	108
4·5	95
5·0	102
5·4	116

Other means of precipitation were tried, *e.g.*, by adding an equal volume of acetone after incubation (Goldstein, 1961). With this concentration of acetone at *p*H 4·5 the supernatant solutions were clear, although there was some difficulty with air bubbles due to the acetone/aqueous mixture. Furthermore, the presence of acetone depressed the phenolphthalein colour to about 30% of that in wholly aqueous solution. When this method was used to study the effect of *p*H upon the enzyme activity it was found that, as in the heat coagulation method, difficulties due to turbidity were encountered at the lower *p*H levels (*p*H 3·6 to 4·0). A compromise was reached in that several *p*H curves were obtained from sera which had previously been shown to have a very slight, or no, turbidity when the protein was not precipitated from the final solution. Also, the protein error was reduced in one case by using serum from a woman 32 weeks pregnant and diluting the highly coloured final solutions with carbonate solution before reading the absorption values. Fig. 1 demonstrates that the *p*H curve in the range 3·6 to 5·6 (in acetate buffer) was substantially similar in the

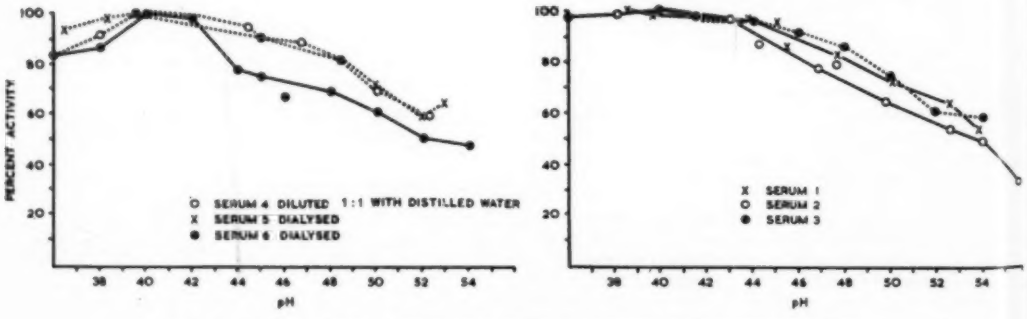

FIG. 1. *Effect of* p*H of the acetate buffer upon serum β-glucuronidase activity.*

various sera and also in dialysed and diluted sera. The curve was flat and in most cases the optimum activity occurred at *p*H 3·9 to 4·0. The activity at *p*H 4·5 was at least 80% of the maximum and so *p*H 4·5 buffer was used in this work.

EFFECT OF DURATION OF INCUBATION The initial velocity was not investigated because of the small quantity of phenolphthalein liberated in less than three hours. Over the range three to 24 hours the amount of phenolphthalein liberated was linear in the sera investigated.

EFFECT OF SUBSTRATE CONCENTRATION AND THE K_m The rate of hydrolysis in 0·2M acetate buffer (*p*H 4·5) increased with increasing substrate concentration over the range 0·25 to 4·0 mM. It was inhibited at higher substrate concentrations. For five different sera the K_m for phenolphthalein mono-β-glucuronic acid, derived by the method of Lineweaver and Burk (1934), ranged from 1·0 to 2·5 mM. The different magnitude of the K_m in different unpurified sera is due to the varying concentrations of substances which influence the enzyme, either inhibitors or activators. In four of the five sera investigated, the K_m was between 2·0 and 2·5 mM. The optimum substrate concentration is theoretically between 4·0 and 5·0 mM. In fact, substrate was inhibited above 4·0 mM in all the sera.

The cost of phenolphthalein mono-β-glucuronic acid is high when obtained commercially and to produce the substance biosynthetically by administering phenolphthalein to rabbits and extracting the glucuronide from the urine is not possible for most hospital biochemical laboratories. Because these considerations were pertinent in the investigation the substrate concentration used was 1·0 mM.

METHOD

About 5 ml. venous blood was taken, allowed to clot, and the serum taken off as soon as possible. The serum was again centifuged in a conical tube to ensure that all cells were removed. Care was taken not to include results from any sample which appeared to the eye to be even slightly haemolysed so that the integrity of the white cells could be assumed in the samples estimated.

Quadruplicate stoppered tubes containing 1·6 ml. 0·2M acetate buffer *p*H 4·5 (11·57 g. sodium acetate ($CH_3COONa{\cdot}3H_2O$) and 6·5 ml. glacial acetic acid to make up to 1 litre), 0·2 ml. serum, 0·2 ml. 0·01M phenolphthalein mono-β-glucuronic acid (Sigma) (510·6 mg./100 ml.) in 10% ethanol were incubated at 37°C. for 18 hours. A blank tube containing the acetate buffer and the serum was also incubated. After 18 hours 0·2 ml. substrate was added to the blank, the tubes were placed in a boiling water bath for five minutes and then cooled in a cold water bath and 2·5 ml. sodium carbonate solution *p*H 10·5 (80 g. $NaHCO_3$ and 150 ml. 20% NaOH made up to 1 litre, the *p*H finally adjusted with NaOH using a *p*H meter) was added to each tube. The coagulum and carbonate solution were carefully mixed by using a narrow glass rod, care being taken not to deposit small pieces of flocculent material on the sides of the tube above the level of the liquid. The solutions were allowed to stand for 10 minutes and then were spun for 10 minutes at 2,500 r.p.m. The supernatant solutions were decanted and read against the blank on a Uvispek spectrophotometer at 550 mμ. The activity was expressed in Fishman units, 1 unit being that which liberates 1μg. phenolphthalein from 1 mM. substrate per hour at 37°C. For comparative purposes the results from normal subjects were also expressed in a standard unit where one unit is that amount of enzyme which will catalyse the liberation of 1 μM. phenolphthalein per minute per litre of serum.

REPRODUCIBILITY Results from aliquot quantities of serum were usually in agreement within ± 5%. Occasionally the differences were greater. There are two main factors which probably contribute to a larger error. Although care is taken to separate completely the cells from the serum it is possible that the incubation solution may at times contain one or more white cells. Another source of error is the possible contamination of the supernatant solution as it is decanted over any protein precipitate which may be adhering to the inside of the test tube above the surface of the solution. This error can be minimized by correct use of the narrow glass rod in mixing the carbonate solution and the protein precipitate.

TECHNICAL FACTORS Samples of cell-free serum could be stored for up to two months at −20°C. or for up to 10 days at 4°C. with a change of activity within ±10%. During the enzyme assay, after coagulation of the proteins, the solution can be left for at least three hours before the carbonate solution is added, without loss of colour intensity. When the alkali is added the colour extraction is complete within 10 minutes and the supernatant should be separated within one hour to prevent errors due to the action of the carbonate solution upon the protein precipitate causing turbidity. The phenolphthalein colour is stable for two hours.

RESULTS

NORMAL VALUES Table V indicates the range of values obtained from 20 men (16 to 60 years) and 21 women (22 to 58 years). The blood was obtained chiefly from people working in various departments of the hospital and also from seven male in-patients before operation such as meniscectomy or herniorrhapy. The mean value for men (680 units/100 ml. serum or 356 standard units) was 62% greater than the mean value for women (418 units/100 ml. serum or 219 standard units). Cohen and Huseby (1951) and Goldbarg *et al.* (1959) noted similar differences.

TABLE V

SERUM β-GLUCURONIDASE LEVELS IN HEALTHY INDIVIDUALS

Men			*Women*		
Age (yr.)	*β-glucuronidase*		*Age (yr.)*	*β-glucuronidase*	
	Units/100 ml. Serum	*Standard Units*		*Units/100 ml. Serum*	*Standard Units*
23	394	206	54	175	92
25	407	213	35	196	103
25	453	237	25	208	109
27	462	242	23	246	129
22	500	262	28	331	173
16	500	262	37	332	174
42	501	263	34	335	176
50	506	265	47	350	183
22	557	292	40	371	194
49	574	301	22	381	200
60	660	346	28	382	200
25	687	360	52	382	200
47	791	415	40	400	210
27	862	452	53	425	223
38	872	457	25	450	236
36	904	474	58	468	245
24	916	481	40	481	252
17	975	511	34	550	288
30	1,030	540	40	568	298
30	1,040	545	57	745	390
			29	994	521
Totals	680	356		418	219

There appeared to be no correlation of enzyme activity with age.

VARIATION DURING THE DAY When blood was taken at intervals during 24 hours the variation in one man and one woman was equivalent to an S.D. of 7% and 4·2% of the mean value (Fig. 2). In another woman blood was taken four times in nine hours and the S.D. of the values was 9% of the mean. In another two patients fasting blood was taken at 8 a.m. and another sample at noon and the differences between the first and second samples were 3% in one patient and 1% in the other.

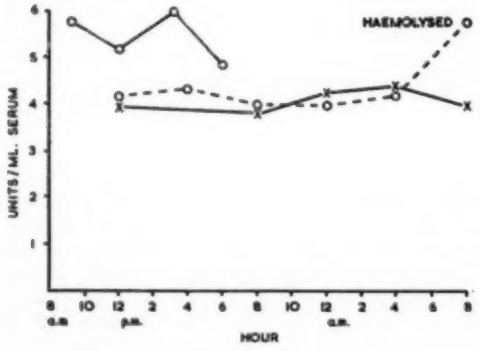

FIG. 2. *Serum β-glucuronidase activity at intervals throughout one day.*

INFLUENCE OF THE MENSTRUAL CYCLE Blood was taken at intervals from four women (age 23 to 40 years) with normal menstrual cycles. The days of the cycle investigated were usually one, five, ovulation day (determined by the rise in waking temperature) 20 and 26, but this timing could not be strictly adhered to. Table VI demonstrates that the greatest variation in the serum β-glucuronidase was in a woman with a standard deviation of 23% of the mean value; in two individuals the standard deviations of the mean were 14% and 17%, and in the fourth, 3%. The variations were therefore relatively small, and there was no common pattern in the differences; only in two individuals was the greatest serum activity during the proliferative phase of the cycle.

TABLE VI

SERUM β-GLUCURONIDASE ACTIVITY DURING MENSTRUAL CYCLE

Units/100 ml. Serum

a[1]	*b*	*c*	*d*[1]
344	250	162	382
(1)[2]	(2)	(1)	(1)
364	187	169	362
(5)	(5)	(5)	(5)
306	258	200	366
(Ov)	(Ov + 1)	(Ov + 1)	(Ov − 1)
325	250	244	375
(20)	(20)	(22)	(21)
344	288	206	382
(26)	(26)	(26)	(26)

[1]For individuals a and d the length of the cycle was 26 days.
[2]Numbers in parentheses indicate the days in the cycle investigated. Ov = ovulation day.

PREGNANCY Table VII indicates that the serum β-glucuronidase level increases during at least the later stages of pregnancy. These few investigations are in agreement with the results presented by McDonald and Odell (1947).

EFFECT OF DIALYSIS OR DILUTION OF THE SERUM In the majority of dialysed sera the activity was enhanced, presumably because dialysible inhibitors were removed. Dilution of serum before assay also results in a proportionally greater activity than in the original serum. When dialysed serum was diluted the relative activity was increased, indicating the presence of undialysible inhibitors.

ENZYME ACTIVITY IN BLOOD CELLS Fishman *et al.* (1948) have reported on β-glucuronidase in blood

TABLE VII

SERUM β-GLUCURONIDASE LEVELS IN PREGNANT WOMEN

Patient No.	*Stage (weeks)*	*β-glucuronidase*	
		Units 100/ml. Serum	*Standard Units*
1	24	950	498
	28	1,175	616
	34	1,890	990
	37	1,900	995
	38	1,875	980
2	29	1,085	568
3	30	1,420	744
4	32	961	504
5	32	1,810	950
6	34	4,650	2,437
7	36	2,240	1,172
8	38	2,180	1,142
9	40	1,825	957

cells where it appears to be concentrated in the leucocytes. The possibility that errors might arise in serum determination due to lysis of the cells has been examined. It has been demonstrated that when whole blood was stored at 4°C. for 24 hours before removal of the serum the serum enzyme value was only ± 6% different from the value in serum immediately separated from the cells. In experiments where red cells and the buffy coat were separated, laked, and estimated, the finding of Fishman *et al.* (1948) that red cells contained little or no enzyme activity although the buffy coat had a large enzyme concentration was corroborated.

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Monitoring sterilization of dressings in high-vacuum pressure-steam sterilizers[1]

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SYNOPSIS Undetected air leaks in a high-vacuum sterilizer can defeat the sterilization process. For this reason the maintenance and supervision of these sterilizers needs expert engineering care. The importance of controlling the high-vacuum sterilization process from the centre of a load rather than the chamber drain is emphasized, and a method of detecting any failure in a high-vacuum process is described. The dynamics of the high-vacuum sterilization process are discussed and the possible disadvantages of an integrator are noted. The wisdom of using a temperature of 134°C. for three minutes is questioned.

Any delay in steam penetrating into a load in a high-vacuum, high-speed sterilizer can defeat the sterilization process. The holding time at 134°C., the temperature of steam at 30 pounds per square inch (p.s.i.), is only three minutes, and although such a rapid process increases the turnover of a sterilizer, the wisdom of having such a short exposure time to steam has been questioned by Knox (1961). In most of the sterilizers at present in use the steaming time is taken from the moment when the chamber drain reaches the temperature of saturated steam at the pressure employed. However, it has been shown (Fallon, 1961) that this temperature may be misleading if steam penetration is delayed due to deficiencies in the fore-vacuum drawn before steam is admitted to a high-vacuum sterilizer. Normally the level of vacuum is monitored so that steam is not admitted to the chamber until a vacuum of 20 mm. Hg absolute pressure or better is reached. Not only could such a monitoring system become faulty but, as has been noted (Fallon, 1961), the fore-vacuum can be defeated by an undetected leak in the sterilizer. The present paper describes experimental work on the effect on steam penetration into a load exposed to steam at 20 or 30 p.s.i. of a known and measurable leak. It deals also with a suitable monitoring system to detect delays in steam penetration due to this or any other cause.

The first indication I had of the importance of a leak in delaying steam penetration into a load in a high-vacuum sterilizer was when I was asked to examine a 60 cu. ft. high-speed, high-vacuum sterilizer which failed to change Browne's tubes No. II (yellow spot) from red to green even with nine minutes' steaming at 134 to 136°C. The fore-vacuum drawn by this machine was found to be 16 mm. Hg absolute pressure even in the presence of a gross air leak. Thermocouples were placed in a load of gowns and towels in a cylindrical dressing drum. In the first experiment (Fig. 1a), in which the thermocouples were introduced round the door of the sterilizer, a remarkable delay in steam penetration into the load was observed. When the thermocouples were placed through a leak-proof fitting the delay was less dramatic (Fig. 1b) but was still extremely serious. The reason for this delay was not clear at that time but when a diaphragm controlling the door-locking mechanism was replaced shortly afterwards, all Browne's tubes placed in loads changed to green quite satisfactorily after a sterilizing run. The probability that the delay was due to an air leak was investigated in an experimental sterilizer.

EXPERIMENT

The 4·2 cu. ft. high-vacuum experimental sterilizer with which this work has been carried out has already been described in detail (Fallon, 1961). The test loads consisted of cotton huck towels measuring 20 in. × 30 in. For most experiments 16 towels were used packed so that they lay in a horizontal plane when placed in the sterilizer, *i.e.*, in a position which would delay steam penetration by downward displacement. Normally they were wrapped in a further two towels to simulate a

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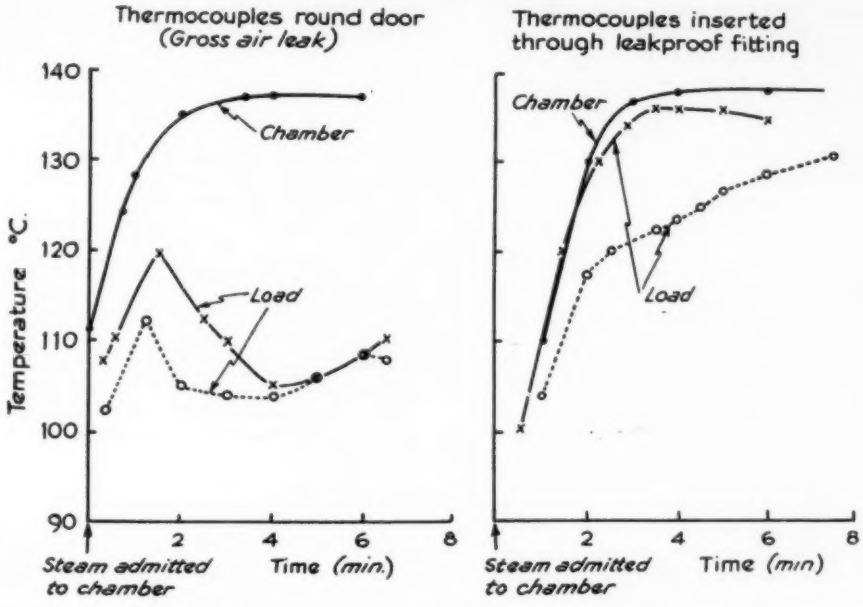

FIG. 1. *Temperature rise inside a 60 cubic foot high-vacuum sterilizer. One thermocouple was free inside the chamber. The other two were placed inside a drum containing gowns and towels.*

drum lining and were then placed in a cylindrical dressing drum 10 in. diameter by 9 in. deep. Results similar to those described below were obtained with only eight towels in a drum or with loads in a cardboard box. The temperature at six standard points in each container was measured, using thermocouples connected to a high-speed electronic recorder.

A small leak was introduced into the sterilizer through the test cock on the door. The leak was one that produced a rise in pressure of 2·5 to 3·5 mm. Hg over a period of one minute when the chamber was evacuated to 20 mm. Hg absolute. The vacuum pump performance was not affected by the presence of such a leak, the pump-down time being the same whether the leak was present or not.

With a leak of this order 43·1 cu. in. (707 ml.) of air leaked into the sterilizer during the period of one minute in which the pressure was raised from 20 mm. Hg absolute to atmospheric by the incoming steam. The effect of such a leak on the temperature rise in a load is shown in Fig. 2. It may be seen that with no leak the rise in temperature inside a load to 126°C. (that of saturated steam at 20 p.s.i.) was rapid and was reflected accurately by the chamber drain thermocouple. Furthermore, during the rise in pressure from atmospheric to 20 p.s.i. the load was always at a temperature corresponding to the pressure of saturated steam in the chamber. With a leak, steam penetration was delayed to a considerable extent and this delay was not shown by the chamber drain thermocouple. Similar results were obtained using steam at a pressure of 30 p.s.i. Slightly larger leaks (approximately 1,100 ml.) caused a much longer delay in steam penetration but also tended to increase the pump-down time.

In order to try to understand just how a leak interferes with steam penetration the following experiment was performed. A fore-vacuum down to 15·8 mm. Hg absolute was drawn. Enough air was then admitted to the chamber to bring the pressure up to 20 mm. Hg absolute. Steam was then admitted to the chamber in the usual way. A gross delay in penetration was observed (Fig. 2). The load was exposed for as long a period to the vacuum as in experiments conducted without any leak of air, in which steam penetrated immediately. The results suggest that a leak does not interfere with steam penetration merely by bringing the effective fore-vacuum to over 20 mm. Hg.

In the 60 cu. ft. sterilizer which first showed this fault, the extensive use of Browne's tubes throughout the load revealed the failure to reach sterilizing conditions. However, it is little consolation to open a 'sterilized' package only to find that the indicator reveals the package not to be sterile; again it will be difficult to place a Browne's tube in every package used in a central sterile supply service. In order to overcome this difficulty a dummy load was devised from which the sterilizing cycle could be monitored and experiments have shown that this gave useful and reproducible results. The load consisted of a

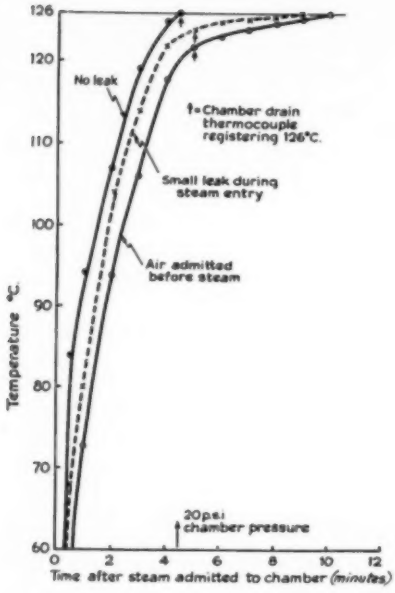
No leak
Chamber drain thermocouple registering 126°C.
Small leak during steam entry
Air admitted before steam
20 p.s.i. chamber pressure
Temperature °C.
Time after steam admitted to chamber (minutes)

FIG. 2. *Slowest rise of temperature recorded by six thermocouples placed inside a load of 16 cotton huck towels.*

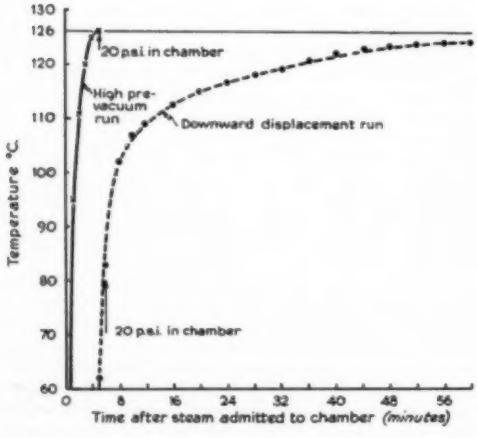

FIG. 3. *Rise of temperature recorded by a thermocouple placed in the middle of the dummy drum.*

10 in. diameter × 4½ in. deep cylindrical dressing drum packed tightly with 1,427 g. of asbestos wool. Asbestos wool was used because, unlike cotton, this would not alter in nature with repeated sterilization and yet it was a fibrous material. A drum was used because this was a convenient container for the fibre. A thermocouple was placed in the centre of the load and the lead entered the drum from the bottom when it was placed on its side in the sterilizer. Other containers, such as wire mesh baskets or a solid box open on one side only, were not successful; nor was a block of compressed asbestos. The load was designed so that it could not be penetrated by steam (using the downward displacement method) in the period of time normally allowed for this to take place, yet it was instantaneously penetrated by steam using a high-vacuum process (Fig. 3). The delay due to the presence of a leak in the sterilizer was well shown by the dummy drum. It was found important to remove the drum from the sterilizer between runs; otherwise heat from the jacket caused superheating on exposure to steam. This would tend to obscure any lag in temperature rise due to a leak.

DISCUSSION

These experiments show that if a high-vacuum sterilizer has a leak which is so small that it can be overcome by the vacuum pump supplied, the air which leaks into the sterilizer, during the period when the pump is switched off and the steam entering the chamber raises the pressure to atmospheric, can seriously delay steam penetrating into a load. This defect may be detected by using large numbers of Browne's tubes, or, perhaps, by observing a delay in drawing the vacuum. It should be detected during a full engineering examination of a sterilizer by using a sensitive vacuum gauge. However, this fault may occur at any time and it may best be detected by monitoring the actual conditions in a load which is difficult to sterilize. The chamber drain temperature does not reflect accurately the conditions in the centre of a load when there is a leak (see Fig. 2) nor when there is an inadequate fore-vacuum (Fallon, 1961), and there can be no doubt that the only way of ensuring that a load has been subjected to an adequate sterilizing process is to record what temperature has been reached at the centre of the load and for how long this temperature has been maintained. The dummy load described above is suitable for this purpose as it can only be penetrated rapidly by steam when an adequate fore-vacuum has been drawn. The dummy load with a permanently fixed thermocouple or other temperature-sensitive device has a further advantage in that it will avoid the possibility of human error in placing the thermocouple into a load in such a way that steam will reach the temperature-sensitive end too easily, either because the thermocouple is placed in the superficial layers of a drum or pack (or even in a very

small container), or because a track is left alongside the thermocouple lead. The thermocouple in the dummy load could not only be connected to a recorder but possibly also to an integrator, such as that described by Bowie (1961), covering a narrow range of time-temperature conditions. In this way the best possible control of the sterilizing process may be achieved. It is possible that two such dummy loads should be used, one for each alternate run, in order that the load should cool adequately between runs.

There is no doubt that a high-speed, high-vacuum sterilizer needs very careful and expert engineering supervision and there is ample room for doubt not only whether such supervision is easily available in all hospitals but whether sterilizers are checked often enough.

The experiment where only enough air was admitted to the chamber to bring the pressure up from 15·8 mm. Hg to 20 mm. Hg absolute throws a light on the dynamics of the high-vacuum sterilizing process. This experiment (which was repeated on several occasions) showed that it was the actual air which leaked in and not the change in absolute pressure which interfered with steam penetration. If the theory is correct that during the high fore-vacuum water is vaporized from the dressings and sweeps out the residual air (*Lancet*, 1959), it may be that not only does the water vapour scavenge air from the load but it may also aid air-scavenging from the chamber by the vacuum pump. When loads of 10 towels were placed in a dressing drum in the sterilizer and exposed to a vacuum falling from 20 to 16 mm. Hg absolute for as short a time as three minutes, it was found that their weight fell by about 5 g. (the load weighing about 860 g.). At a pressure of 15·8 mm. Hg absolute and a temperature of 18·3°C. (65°F.) this amount of water, as vapour, would occupy a volume of 11·26 cubic feet (318·9 litres) and obviously the generation of such an amount of water vapour would aid air-scavenging from the sterilizer. If it is remembered that 10 towels represent a very small load and that the temperature within the chamber (in a sterilizer with a heated jacket) will be over 18°C. in normal circumstances, it can be seen that this effect may be very important. When this scavenging of air is defeated by a leak, the air that is in the chamber may well be carried back into the load as the incoming steam rushes in to condense on the vast surface of the fibres of the dressings. This hypothesis explains not only what happens when a leak allows air to get back into the chamber but also why it is essential to hold the vacuum and not merely reach a level of 20 mm. Hg and then immediately let steam into the chamber (Bowie, 1961). As noted in the M.R.C. report (*Lancet*, 1959), an absolute pressure of 20 mm. Hg is above the level at which water will boil at room temperature and it is not easy to see why the level of 20 mm. Hg should be an adequate fore-vacuum. The answer probably lies in the fact that the chamber will be much hotter than room temperature and also that some vacuum pumps will pump to a level below 20 mm. Hg. absolute during the holding period of vacuum allowed for on some commercial models. The hypothesis would also explain why inadequate degrees of fore-vacuum, *i.e.*, above 20 mm. Hg absolute, result in a delay in steam penetration into a load (Knox and Penikett, 1958; Fallon, 1961).

The observation that a leak delays steam penetration into a load and the possible explanation for this noted above raises the question of the value of an integrator on a sterilizer in the presence of a leak or faulty fore-vacuum. In a normally functioning high-vacuum sterilizer the time-temperature integrator can be brought into action as soon as the load reaches, say, 121°C., as this temperature is due to the presence of saturated steam which will sterilize at this temperature. The time the load is above this temperature may be computed by an integrator until an adequate sterilizing period has been achieved. If, however, there is a leak, the rise in temperature in the load is delayed compared with the temperature in the chamber and this clearly must be due to the presence of air. In this case it is not permissible to integrate the time-temperature conditions until the load reaches the temperature corresponding to the pressure of steam in the chamber. Hence, although an integrator is a useful addition to a perfectly functioning sterilizer, it is clearly vital that a monitoring system should also be attached to a recorder so that any fault in the sterilizing process will be evident to the sterilizer supervisor who can then take appropriate action.

In view of the many snags that can arise, the wisdom of using a temperature of 134°C. (30 p.s.i.) for three minutes as a sterilization process should be carefully reconsidered because the margin of safety is extremely small and may be unobtrusively exceeded by quite small leaks of air.

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Four cases of human infection with *Achromobacter anitratus*

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SYNOPSIS Four cases of human infection with *Achromobacter anitratus*, one of which was fatal, are described. They all occurred at one hospital within a period of two months.

The bacteriology, antibiotic sensitivity, and pathogenicity of the organisms are discussed. One of the strains was found to differ from the other three.

Achromobacter anitratus is rarely reported as the causative organism in human infections. We could find only six fatal cases described in the literature, four of them in one article (Lutz, Grootten, Velu, and Velu, 1956) and the other two individually (Ferguson and Roberts, 1950; Gardner, Pines, and Stewart, 1960). We record four instances of infection with this organism, one of which was fatal, from the practice of one hospital during a period of two months.

CASE REPORTS

CASE 1 A man of 55 was admitted urgently suffering from severe abdominal pain and nausea. There was no history of dyspepsia or of previous similar attacks of pain. On examination the abdomen was as rigid as a board and he was noted to have generalized neurofibromatosis. At laparotomy there was free bile-stained fluid in the abdominal cavity and generalized peritonitis. No cause for the peritonitis was found, there was no peptic ulcer and no perforation of the bowel, the appendix was healthy, and, apart from containing a few small stones, the gall bladder appeared normal. There were no herniae and no diverticulae of the bowel. The abdomen was closed with drainage; the drainage fluid contained large amounts of amylase and some bile and the serum amylase content was high (1,000 units); these results suggested a biliary and pancreatic fistula. The condition of the patient deteriorated despite supportive and antibiotic therapy and one month after the operation he died. *Achromobacter anitratus* associated with *E. coli* was isolated on several occasions from the drainage fluid. Post-mortem examination was carried out the day after death. The principal findings were as follows:—

There was generalized neurofibromatosis affecting the superficial nerves of the skin and also the nerves of the abdominal viscera. There was an abscess in the lesser sac of the peritoneum. The liver was enlarged and contained many micro-abscesses which were shown on histological section to be related to ascending infection of the bile ducts. The pancreas was firm but not enlarged; there were no areas of fat necrosis. The pancreatic ducts were dilated and there was a fistula between a major pancreatic duct and the abscess cavity in the lesser sac. There was a neurofibromatous nodule in the duodenal mucosa adjacent and just proximal to the ampulla of Vater, compressing the pancreatic and bile ducts.

There was a moderate degree of chronic bronchitis and emphysema. The heart was normal, but there was well marked atheroma in the cerebral and coronary vessels. *Achromobacter anitratus* was isolated in pure culture from the liver, the subphrenic abscess, and the abdominal sinus. This organism was not isolated from the blood, the general peritoneal cavity, or from the lungs.

CASE 2 A woman aged 75 was admitted to hospital for treatment of a fracture of the neck of the right femur. The fracture was reduced and secured by internal fixation; 14 days after operation superficial infection of the operation wound was noted and she also had a small bedsore. Infection was confined to the most superficial tissues and it was at no time necessary to re-open the wound. Local treatment with acriflavine in spirit was used and both the bedsore and the wound infection healed completely before the patient was discharged from hospital.

Achromobacter anitratus was isolated from the operation wound and from the bedsore, in each instance in association with coagulase-negative staphylococci.

CASE 3 A man aged 23 was admitted suffering from an abscess in the superficial tissues of the perineal region. The lesion was draining at the time of admission. It was treated by local applications of kaolin poultices and dressings and healed satisfactorily.

Achromobacter anitratus was isolated in pure culture from this abscess.

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CASE 4 A man aged 41 was admitted for arthrodesis of the ankle after a compound fracture which had led to painful arthritis. On admission he was found to have a minor infection of the old wound and operation was deferred. *Achromobacter anitratus* was isolated in pure culture from this lesion, which healed without event.

DESCRIPTION OF ACHROMOBACTER ANITRATUS

All four strains were small Gram-negative coccobacilli possessing capsules. All were non-motile. All grew well on unenriched media and MacConkey agar at room temperature and 37°C. but did not grow on deoxycholate-citrate agar or S.S.agar. There was slight haemolysis on horse-blood agar. No growth occurred anaerobically. On nutrient agar after 24 hours at 37°C. the colonies were circular, about 1 mm. in diameter, convex, smooth and glistening with an entire edge; they were greyish white, moderately opaque, iridescent, of a butyrous consistency and emulsified easily. Detailed biochemical reactions are given in Table I.

TABLE I

BIOCHEMICAL REACTIONS OF STRAINS OF *ACHROMOBACTER ANITRATUS*

	Case 1	*Case 2*	*Case 3*	*Case 4*
Glucose	A	A	A	A
Lactose	–	–	–	–
10% lactose agar	A	A	A	A
Sucrose	–	–	–	–
Salicin	–	–	–	–
Maltose	–	–	–	–
Mannitol	–	–	–	–
Galactose	A	A	A	A
Arabinose	A	A	A	A
Xylose	A	A	A	A
Rhamnose	–	–	–	–
Dulcitol	–	–	–	–
Salicin	–	–	–	–
Indole	–	–	–	–
Methyl red	–	–	±	–
Voges Proskauer	–	–	–	–
Citrate	+	+	+	+
Urease 24 hours	–	–	–	–
7 days	+	+	–	+
Oxidase	–	–	–	–
Hydrogen sulphide	–	–	–	–
Nitrate	–	–	–	–
Gelatin	–	–	+	–
Growth at 44°	+	+	–	+
Soap tolerance	–	–	+	–

– = no reaction
± = weak positive reaction
A = acid produced in 24 hours

The sugar reactions were carried out in peptone water with 1% added sugar with 0·01% phenol red indicator; incubation was at 37°C. and all tubes were kept for 21 days. The 10% lactose agar medium recommended by Chilton and Fulton (1946) was used in addition. The indole, methyl red, Voges Proskauer, oxidase, and citrate utilization tests were carried out by the techniques of Mackie and McCartney (1960). Urea hydrolysis was tested on the medium described by Christensen (1946); the tubes were incubated at 37°C. and examined after 24 hours and seven days. Hydrogen sulphide production was tested in Kligler's iron agar medium. Nitrate reduction was tested both in broth culture (Wilson and Miles, 1955) and by a plate method (Cook, 1950). Gelatin liquefaction was investigated by growing the organism in this medium at room temperature. Growth at 44° was tested in nutrient broth, and the soap tolerance was tested on 10% soap agar plates (Billing, 1955).

ANIMAL PATHOGENICITY

Animal pathogenicity was determined in both mice and guinea-pigs. An overnight broth culture was made and 0·5 ml. was inoculated intraperitoneally into each of four mice and 1 ml. into each of two guinea-pigs. Strains from Cases 1 and 4 were pathogenic for both mice and guinea-pigs, the animals dying within 24 hours; large numbers of *Achromobacter anitratus* were recovered from the peritoneal cavity and spleen and heart blood. The strain from Case 2 was pathogenic for mice but not guinea-pigs and the strain from Case 3 was not pathogenic for either of these species. The strain from Case 3 differed from the other three strains in its absence of pathogenicity, its ability to liquefy gelatin, its tolerance of soap, and its failure to grow at 44°C. This strain was agglutinated by a serum prepared at the Central Public Health Laboratory against a gelatin-liquefying strain of *Achromobacter anitratus* isolated in 1953 from an abscess. This serum did not agglutinate the strains isolated from Cases 1, 2, and 4. Billing (1955) stated that her gelatin-liquefying, soap-tolerant strains were not pathogenic, although two out of her 31 strains were obtained from pathological material. Our strain was isolated in pure culture from an ischio-rectal abscess, which suggests that it was mildly pathogenic. Its absence of pathogenicity for laboratory animals does, however, suggest a pathogenicity lower than that of the other strains.

ANTIBIOTIC SENSITIVITY

Sulphonamide sensitivity was tested on lysed 5% horse blood agar using a commercial disc. Determination of the antibiotic sensitivities was carried out on nutrient agar plates also using commercial discs. The results are summarized in Table II. Table III shows the variability of the results of sensitivity on *Achromobacter anitratus* reported in the literature (Moor, 1949; Brooke, 1951; Waage, 1953; Lund, 1954; Lutz *et al.*, 1956; Gardner *et al.*, 1960). Most strains are resistant to sulphonamides and to the antibiotics penicillin and chloramphenicol;

TABLE II

ANTIBIOTIC SENSITIVITY OF *ACHROMOBACTER ANITRATUS*

	Case 1	*Case 2*	*Case 3*	*Case 4*
Penicillin (1·5 units)	Resistant	Resistant	Resistant	Resistant
Streptomycin (10μg.)	Resistant	Resistant	Sensitive	Resistant
Chloramphenicol (10μg.)	Resistant	Resistant	Resistant	Resistant
Tetracycline (10μg.)	Resistant	Resistant	Sensitive	Resistant
Bacitracin (5 units)	Resistant	Resistant	Resistant	Resistant
Erythromycin (10μg.)	Sensitive	Sensitive	Sensitive	Resistant
Neomycin (10μg.)	Sensitive	Sensitive	Sensitive	Sensitive
Polymyxin B. (10μg.)	Sensitive	Sensitive	Sensitive	Sensitive
Sulphonamide (100μg.)	Resistant	Resistant	Resistant	Resistant

TABLE III

ANTIBIOTIC SENSITIVITY OF *ACHROMOBACTER ANITRATUS*

	Moor (1949)	*Brooke (1951)*	*Waage (1953)*	*Lund (1954)*	*Lutz et al. (1956)*	*Gardner, Pines, and Stewart (1960)*	*Ashley and Kwantes (Present Series)*
Strains	15	86	1	77	7	1	4
Sulphonamide	R	S 15 R 71	R	60% R		S	R
Penicillin	R	S 1 R 85	R	R	R	R	R
Streptomycin	S	S 43 R 43	S	39% R		R	1 S 3 R
Chloramphenicol		S 1 R 85	S	95% R	R	R	R
Chlortetracycline		S 81 R 5	R	S		R	
Tetracycline		S 76 R 10	R	98% S	Variable		3 R 1 S
Erythromycin				88% R	R		3 S 1 R
Bacitracin				R	R		R
Neomycin				S	S		S
Spiramycin					R		
Polymyxin B					S		S

sensitivity to streptomycin and the tetracycline group is variable and there appears to be uniform sensitivity to neomycin and uniform resistance to bacitracin.

EPIDEMIOLOGY

These cases occurred independently of each other; all were in different wards of the hospital and were under the care of three separate surgeons. One surgeon had two of the patients under his care, but these were admitted at an interval of nearly two months to widely separated wards. There were no nurses or doctors common to any of the patients who were in hospital at the same time and there was no known contact between the patients outside the hospital before admission.

DISCUSSION

Achromobacter anitratus is a little known organism belonging to the tribe *Achromobactereae*. Schaub and Hauber (1948) examined 15 strains and reported on the biochemical and serological characteristics of the organism. They proposed the name *Bacterium anitratum*. Further consideration of the taxonomy of this group of organisms has led to the adoption of the currently accepted name *Achomobacter anitratus* (Billing, 1955).

The organism is a Gram-negative bacillus, usually coccobacillary in form, and often resembling organisms of the genus *Neisseria* from which it may be distinguished by the negative oxidase reaction. It closely resembles the organism described by von Lingelsheim (1906, 1908) who isolated from the cerebrospinal fluid an encapsulated organism which he called *Diplococcus mucosus*. Several authors have isolated further strains of *Diplococcus mucosus* from specimens of cerebrospinal fluid and have noted that they were capable of growth on MacConkey agar (Cowan, 1938; McFarlan, 1941). Seeliger (1953) regards the recently recognized *Achromobacter anitratus* and *Diplococcus mucosus* as identical but the ability of the strains of the latter organisms isolated by Cowan (1938) to reduce nitrates suggests a separate identity.

Piéchaud, Piéchaud, and Second (1951, 1956) included this organism in the genus *Moraxella* and renamed it *Moraxella glucidolytica* whereas Ewing (1949) proposed its inclusion in the tribe *Mimeae* genus *Herellea* (De Bord, 1939, 1942). Henriksen (1952, 1960) has reviewed strains of *Achromobacter anitratus* from the viewpoint of taxonomy. He showed that representative strains of this organism have little resemblance to members of the genus *Moraxella* and placed them in the tribe *Achromobactereae*. He also mentioned the resemblance of *Achromobacter anitratus* to the *Herellea* genus but questioned the validity of the tribe *Mimeae*.

Despite its unusual nature *Achromobacter anitratus* is easily identified. It appears on primary isolations or on subculture as a non-lactose fermenting colony on MacConkey's medium with a characteristic cocco-bacillary morphology. Further confirmation of its identity is made by the sugar reactions; it produces acid in glucose but, as it is strictly aerobic, the fermentation is seen to be greater in the upper part of the tube and there is no fermentation at all in the Durham's tube. The routine sugars—mannitol, lactose, sucrose, and dulcitol—are not fermented and no indole is produced. Finally its ability to utilize citrate, the negative oxidase reaction and its failure to reduce nitrates confirm its identity. For this last test we have found the nitrate plate described by Cook (1950) of great value.

The natural habitat of the organism is uncertain. Isolations have been made from faeces and urine of apparently normal people (Philpot, 1956; Billing, 1955) and a variant tolerant of soap has been isolated from face flannels and sponges (Billing, 1955). It is usually regarded as of low pathogenicity (Wilson and Miles, 1955) but a number of instances of human infection have been recorded and strains have been found to be pathogenic to mice, guinea-pigs, and rabbits (Schaub and Hauber, 1948; Stuart, Formal, and McGann, 1949; Lutz *et al.*, 1956, and the present series) although contrary findings have also been recorded (Brooke, 1951; Waage, 1953).

Stuart and Van Stratum (1945) isolated *Achromobacter anitratus* from the faeces of 10 infants involved in a small outbreak of gastroenteritis and further association with gastroenteritis was noted by Stuart *et al.* (1949). It must, however, be remembered that at this time the role played in the aetiology of gastroenteritis by the 'specific' strains of *E. coli* was not recognized and these isolations may have been of commensal organisms. Other reports of human infection with this bacterium have covered a wide range of clinical disorders. In most cases the report was directed to the bacteriology of the strain and clinical data are scanty. One common feature, however, has been the concurrent isolation of other organisms, often of the coli group (Rocha and Guze, 1957). Moor (1949) described a series of 15 cases of conjunctivitis from which *Achromobacter anitratus* was isolated in pure culture. He noted that several of the cases had occurred among patients consulting the same ophthalmologist and suggested the possibility of cross-infection. Inoculation of cultures of the organism into the eyes of rabbits induced a mild conjunctivitis from which, however, no bacterial growth was obtained and similar experiments on cats showed no pathogenic effect.

There have been several fatalities following infection of the meninges after cranial operation (Lutz *et al.*, 1956). A recent fatal case was described in 1960 by Gardner *et al.* Their patient had suffered from chronic bronchitis for many years and died rapidly from a fulminating pneumonia and septicaemia. *Achromobacter anitratus* was isolated in a heavy growth from the sputum and was seen in smears of bronchial pus at necropsy. Case 1 is another fatality recorded of infection with this organism. In all of these cases infection with *Achromobacter anitratus* has supervened upon a serious pre-existing disease such as chronic bronchitis or chemical peritonitis due to the release of biliary and pancreatic enzymes into the peritoneum or upon a cranial operation. This association with other disease is seen also in the non-fatal cases recorded. The three patients of Rocha and Guze (1957), in whom the organism appeared to play a major role, were suffering from cirrhosis of the liver, operation for closure of a patent ductus arteriosus, and renal calculus associated with severe paralytic poliomyelitis respectively. These observations and the common association of *Archromobacter anitratus* with other organisms suggests that the organism is of low pathogenicity towards man.

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Technical methods

Control material for oxalo-acetic transaminase determination

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The estimation of serum transaminase activity is routinely performed in many laboratories, and, although serum of high activity has been recommended as control material, we have found it of limited value owing to instability for any period longer than a few weeks and because of vagaries of supply. We felt, therefore, that a more stable material was required to control these determinations over longer periods. With this end in view we prepared extracts of cardiac muscle according to the method of Cammarata and Cohen (1951) as far as precipitate WFT-11, omitting the final stages of purification, and studied its activity under varied conditions of storage.

The final yield amounted to approximately 1·0 g. of precipitate from 1,000 g. of wet heart muscle. The precipitate was prepared for storage in two forms. (1) Ampoules were made containing 28 mg. of freeze-dried material each, which was reconstituted with 20 ml. distilled water (1 in 714 wt./vol.) giving a solution with a transaminase activity of 60 units/ml. (2) Aqueous solutions with various convenient levels of enzyme activity were made from the precipitate and these were stored as below.

STORAGE OF EXTRACT

The following conditions of storage were studied:—

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1 Container, polystyrene or glass (Table I A); 2, concentration of extract, high or low (Table I B); 3, temperature, frozen (−20°C.), cooled (+4°C.), and room (Table I C); 4, freeze-dried and stored at room temperature and reconstituted with water before use (Table II).

TABLE II

RESULTS FOR FREEZE-DRIED EXTRACT

Weeks	*Activity as Percentage of Original*
0	100
4	94
16	100
20	100
26	94
36	102

Using Sigma prepared reagents and subsequently the method of Reitman and Frankel (1957), the oxalo-acetic transaminase activity was determined at frequent intervals on the material extracted. A selection of the relevant results is shown in the accompanying tables. All results are expressed as a percentage of the original activity.

DISCUSSION

Cammarata and Cohen observed deterioration of activity over a six-week period with their final lyophilized enzyme, but using the less purified preparation we obtained a stable product for at least nine months when it was stored in the lyophilized state at room temperature.

Extract of cardiac muscle was found to be suitable, for approximately six months, as control material for oxalo-acetic transaminase determinations when stored in polystyrene containers at high concentrations at −20°C

TABLE I

RESULTS AFTER STORAGE

Weeks	*Activity as Percentage of Original*						
	A *Both 4,000 units/ml. at −20°C.*		*B* *Both in Polystyrene at −20°C.*		*C* *All 8,000 units/ml. Polystyrene*		
	Polystyrene	*Glass*	*8,000 units/ml.*	*40 units/ml.*	*−20°C.*	*+4°C.*	*Room Temperature*
0	100	100	100	100	100	100	100
1	96	96	94	100	94	94	77
2	98	94	94	72	94	94	66
4	100	86	102	47	102	100	49
16	96	70	94	—	94	100	—
26	90	64	102	—	102	70	—
36	78	60	77	—	77	—	—

Freeze-dried extract stored at room temperature is suitable for at least nine months.

We should like to thank Dr. P. J. Sewell for his help with the freeze-drying of the extract.

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The extraction of Evans blue from plasma

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It is possible to extract Evans blue quantitatively from plasma by the following procedure. Unfortunately, haem pigments and the yellow pigments of plasma are also extracted and make a substantial contribution to the absorption at the wavelength of the Evans blue absorption band but this can be corrected by a blank determination.

Evans blue forms a complex with benzalkonium chloride which can be extracted from the aqueous phase with *n*-butanol. Proteins are precipitated and after centrifugation form a tough layer at the interface. Since water and *n*-butanol are mutually soluble, the benzalkonium reagent is saturated with butanol, and kept at + 4°C., thus ensuring that even at the end of the extraction process the temperature is slightly below ambient. If during centrifugation the temperature is allowed to rise above ambient on cooling the extract separates out into two phases and becomes turbid.

REAGENTS

Fifty per cent benzalkonium chloride U.S.P. (Marinol, Bayer Products) is diluted 1 in 8 with distilled water; a drop of concentrated hydrochloric acid is added to each 100 ml., and enough *n*-butanol to saturate it at +4°C., approximately 8 ml. per 100 ml. This reagent and *n*-butanol (A.R.) are kept in a refrigerator at 4°C.

PROCEDURE

This procedure is designed for the Unicam SP 350 or Coleman junior absorptiometer or other instrument requiring 2·5 to 2·8 ml. samples.

Place 5 ml. benzalkonium reagent in a centrifuge tube and add 2 ml. dyed plasma and 3 ml. *n*-butanol. Cover with Parafilm, shake vigorously, and centrifuge at 3,000 r.p.m. for four to five minutes. Transfer the clear supernatant to a clean tube or directly to the cuvette, and read against a *n*-butanol blank at 605 mμ.

BLOOD VOLUME DETERMINATIONS

It is essential to prepare a blank from a sample collected before the dye is injected. It is not possible to correct by using measurements at 405 mμ, or to remove the interfering pigments.

I am indebted to Messrs. Bayer Products, Ltd., for gifts of benzalkonium chloride and of other alkyl benzyldimethylammonium chlorides.

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Book reviews

A CIBA FOUNDATION SYMPOSIUM ON CELLULAR ASPECTS OF IMMUNITY. Edited by G. E. W. Wolstenholme and Maeve O'Connor. (Pp. xii + 495; 117 figures. 60*s*.) London: Churchill. 1960.

Many distinguished names in the field of immunology have contributed to this, the most recent volume sponsored by the Ciba Foundation, which represents the proceedings of a symposium on problems concerning the cellular aspects of immunity. It contains many thought-provoking concepts of the immune state as interpreted in the light of recent experimental findings, and not the least stimulating facet of the lavishly illustrated book are the discussions which follow each chapter. The significance of the central role played by the immunologically competent cell in immunology has been duly stressed by the contribution of Simonsen, Bernhard, Thiéryl and Dixon.

There is a valuable chapter by Medawar summarizing the obstacles which beset the formation of an adequate theory of immunological tolerance and indicating the lines for future research in this field. The phenomena of the delayed hypersensitivity and homograft reactions, the cellular origins of the serum proteins related to immunity, the properties of transfer factor, and studies on antibody formation are discussed in the light of recent findings.

The book also affords a valuable summary of the expansive literature on immunology and is recommended to all who are interested in the subject.

FRANCIS MARTIN

A LABORATORY HANDBOOK OF BLOOD TRANSFUSION TECHNIQUES. By A. Derek Farr. (Pp. xi + 135; 34 figures. 17*s*. 6*d*.) London: Heinemann. 1961.

The author of this small book sets out to describe the practice of blood transfusion as seen from the laboratory point of view. He described the details of preparing the apparatus, the anticoagulant solutions, and the types of collecting and giving sets. The techniques of sterilization of both the solutions and the apparatus are well documented as are the sterilization control methods.

The whole sequence of events from selection of donors, collection of blood, storage, blood grouping, compatibility testing, administration, and possible transfusion reactions, is adequately treated.

It is well written in a fresh, terse style, while the clear and well-chosen illustrations enhance the value of the text.

The book is not intended as a complete compendium on the subject of blood transfusion techniques, but rather as a companion to a work on blood transfusion serology. Those engaged in the transfusion service will find this volume useful, but because it lacks the detail of transfusion serology, it cannot be regarded as a complete manual for workers in hospital blood banking, but in its chosen field, it achieves its aim of providing authoritative information on the laboratory aspects of blood transfusion. It would appear to be written mainly for technicians and for them it provides an accurate and helpful source of useful information at modest cost.

M. G. NELSON

CHROMATOGRAPHIC AND ELECTROPHORETIC TECHNIQUES. Edited by Ivor Smith. Vol. I: Chromatography, 2nd ed. (Pp. xxii + 671; 177 figures. 65*s*.); Vol. II: Zone Electrophoresis, 1st ed. (Pp. viii + 215; 186 figures. 30*s*.) London: Heinemann. 1960.

The appearance of a new and much expanded edition of this work, less than three years after the original, testifies both to its popularity and to the rapid progress being made in this field. A quick glance at the list of contents is enough to show that chromatographic procedures now have their applications in nearly all spheres of biochemical investigation. New chapters have been devoted to such topics as dinitro-phenyl aminoacids, iodoaminoacids, plant phenols and tannins, phospholipids, barbiturates, and glutarimides, and the earlier chapters have been expanded and brought up to date. The sections of aminoacids, sugars, indoles, and steroids are of particular value to the hospital laboratory worker, but it may only be a matter of time before chromatography of other chemical families is found to have diagnostic importance.

A companion volume on zone electrophoresis successfully follows the object of the original work, *i.e.*, the emphasis throughout is practical; instructions are given to the last detail. It deals with paper electrophoresis of proteins (including haemoglobins, lipo and glycoproteins), aminoacids and nucleotides, and there are sections on the use of other media such as agar, starch, and perhaps most important, cellulose acetate. Because there are many authors there is unfortunately a good deal of repetition in this volume, an obvious though not a serious defect which should not be difficult to eradicate in a future edition. In these days when new books so often share the same subject matter, it is a pleasure to find one which has probably no equivalent for British workers, for whom it is well on the way to becoming a standard authority.

WM. H. R. AULD

TECHNIQUES IN TROPICAL PATHOLOGY. By B. G. Maegraith, W. E. Kershaw, and D. Dagnall. (Pp. xi+164; 6 plates, 4 text figures. 25*s*.) Edinburgh and London: Oliver & Boyd. 1961.

This handbook is intended for use in small laboratories in the tropics by medical practitioners on the one hand and by technicians with some elementary training in routine laboratory methods on the other.

In attempting to meet the needs of both types of person it has failed to meet the needs of either. The descriptions of techniques lack balance both in choice and content and there are some omissions, *e.g.*, syphilis. This book is not recommended.

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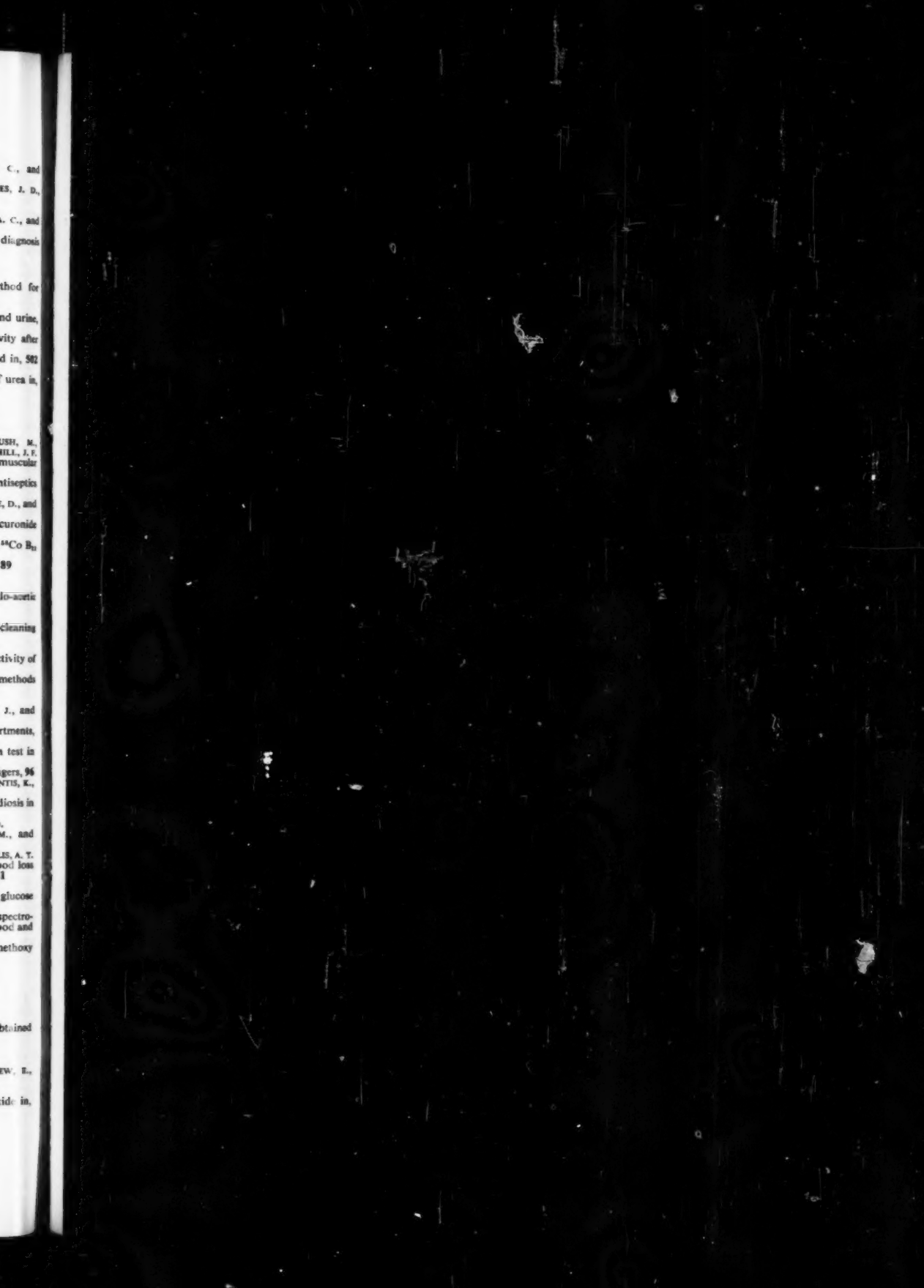
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